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13. ABSTRACT (Maximum 200 Words)

Antiestrogens are the most effective and widely administered therapy for the management of breast cancer. Their efficacy has been attributed to their ability to antagonize the estrogen receptor, and the presence of ER in breast tumor biopsy specimens correlates well with responsiveness to antiestrogen therapy. Still, one in four patients with ER-positive tumors do not respond to antiestrogens, while one in six patients with ER-negative breast tumors undergo objective tumor regression following antiestrogen therapy (Witliff, 1984). These clinical observations suggest that alternative mechanisms of estrogen action may regulate the growth and survival of breast tumors. We have provided evidence that estrogen acts independently of the known estrogen receptors, ERα and ERβ, via the G-protein coupled receptor, GPR30, to regulate the EGFR-to-MAP K signaling axis (Filardo et al, 2000; reviewed in Filardo, 2001; Filardo et al, 2001). Moreover, we have shown that the antiestrogens, tamoxifen and faslodex (ICI 182, 780), also trigger GPR30-dependent regulation of this HB-EGF autocrine loop.

Dysregulation of the EGFR-to-MAP K signaling axis is a common occurrence in breast cancer (Slamon et al, 1989, Sivaraman et al, 1997). The subject of this DOD award is to investigate the relationship between GPR30 expression and MAP K activity in breast tumor biopsy specimens obtained at first diagnosis or following antiestrogen or other adjuvant therapies. The results of these studies may lead to a further refinement in assessing responsiveness to antiestrogen therapy.

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INTRODUCTION.

Tamoxifen is the most effective and widely administered drug for the treatment of breast cancer, providing improved disease-free and overall survival for approximately 40% of breast cancer patients (Gradishar and Jordan, 1998). More recently, it has been shown that tamoxifen also serves as a chemopreventive agent for certain women at high risk for developing breast cancer (Fisher et al, 1998). Its value as a therapeutic agent is presumed to be associated with its ability to function as an ER antagonist (Katzenellenbogen et al, 1997), and accordingly, the presence of ER in breast tumor biopsy specimens provides some degree of certainty in terms of predicting responsiveness to tamoxifen therapy (Witliff, 1984). However, one in four patients with ER-positive tumors (15% of all breast tumors) for whom tamoxifen is indicated, fail tamoxifen therapy; and one in six patients with ER-negative tumors (5 % of all breast tumors), for whom tamoxifen is not currently indicated, exhibit objective tumor regression (Witliff, 1984). Moreover, it is not uncommon for breast carcinomas that initially respond to tamoxifen to acquire tamoxifen resistance, and eventually fail treatment (Katzenellenbogen et al, 1997). Thus, there is an urgent need to identify patients capable of responding to tamoxifen therapy from those who require alternative treatment modalities. This is particularly true for patients with ER-negative tumors since the majority of these patients do not respond to tamoxifen, and consequently, they have fewer treatment options.

GPR30, an orphan G-protein coupled receptor, transmits intracellular signals that regulate the mitogen-activated protein kinases, Erk-1 and Erk-2 in breast cancer cells following tamoxifen treatment (Filardo et al, 2000, Filardo et al 2002, reviewed in Filardo et al, 2002). Erk-1/-2 hold particular significance for breast cancer because these kinases are commonly hyperactivated in breast carcinoma (Sivaraman et al, 1997); and they are key signaling intermediaries for both estrogen and growth factor-dependent pathways.

BODY.

Our hypothesis is that low levels of GPR30 expression, or somatic mutations within GPR30, may compromise the ability of tamoxifen to regulate Erk-1/-2 activity, and correlate with nonresponsiveness to tamoxifen therapy. In the work supported by this award, we are testing this hypothesis by comparing the expression of GPR30 and Erk-1/-2 activity in archival breast biopsy specimens obtained at first diagnosis (prior to treatment) or subsequent to adjuvant therapy.

GPR30 expression in normal human tissue.

To evaluate whether GPR30 serves as an indicator for assessing antiestrogen responsiveness, we have generated rabbit antibodies directed against peptides derived from the deduced amino acid sequence of GPR30 to evaluate the expression of GPR30 protein in archival, formalin-fixed, tissue specimens.

Survey of normal human tissues.
While the work outlined in the original proposal does not specifically state that we would investigate GPR30 expression in tissues other than breast, this information is vital to the assessment of GPR30 as an estrogen-responsive marker. To this end, we have evaluated GPR30 expression in commercially available tissue microarrays (TMAs) (Ambion Inc) that contain a multitude of normal human tissues. The results of this survey are presented in Table I, with some representative examples shown in Figures 1 and 2.

In general, our survey indicates that GPR30 is not ubiquitously expressed. High levels of GPR30 are detected in female and male reproductive tissues, including mammary epithelia (figure 1A) and seminal vesicles (figure 2B). Testis and prostate (figure 2A) also express strong levels of GPR30. More modest levels of expression are observed in kidney (figure 2C) and in pancreas. Hematopoietic tissues (spleen, tonsil, and lymph node) are negative. As is skeletal muscle, heart, liver and thyroid.

These data are likely to indicate that GPR30 action may occur only in a subset of human tissues, and provide further specificity regarding the quality assurance of our peptide antibodies.

GPR30 expression in human breast tumor biopsies.
Preinvasive breast cancer. We reported last year that GPR30 expression is common in normal mammary epithelia and in preinvasive breast cancer (figure 3). During this past year, by making use of TMAs from the National Cancer Institute Cooperative Breast Cancer Tissue Resource (CBCTR), we have extended our analysis of preinvasive breast cancer cases. These results are summarized in table II, and include prior cases that we have analyzed from Brown University Medical School-affiliated Hospitals.

To date, we have examined 20 cases of preinvasive breast cancer, including apocrine metaplasia, benign hyperplasia, atypical ductal hyperplasia (ADH) and ductal carcinoma in situ (DCIS). In the majority of cases (18/20) the level of GPR30 expression detected is commensurate with that measured in normal mammary tissue. We did detect 2 cases of ductal carcinoma in situ (DCIS) where GPR30 was not detected. Further numbers are required, and will be obtained from the NCI CBCTR to fully evaluate GPR30 expression in preinvasive breast cancer.

Invasive ductal carcinoma. Again by use of TMAs from the NCI, we have greatly increased the number of tumor specimens that we have been able to attain for the analysis of GPR30 expression. To date we have stained, 376 cases of invasive ductal carcinoma for GPR30 expression. The value of the CBCTR TMAs is that they are provided in a double- blind fashion. Upon receipt of our scoresheet registering GPR30 expression in each of the grids of the tissue microarray, the NCI sends back to us a detailed analysis of each sample. Included with the histopathological analysis of the samples are clinicopathological markers, such as tumor size, node involvement and steroid hormone receptor status.

At the time of this report, clinical data has been received for 188 of the invasive ductal carcinoma cases, and these data are correlated with GPR30 expression in table III. In general, we have not observed a correlation between GPR30 expression, tumor size, node involvement or steroid hormone receptor status. However, we have observed heterogeneity in the intensity of GPR30 expression in these cases that appears to be independent of clinical parameter. For example, approximately 30% (23/75) of the tumors that we have examined that are less than 2 cm lack GPR30, with the remaining 70% of these samples registering as positives on a scale of +1 to +3. 25% of the node negatives (12/50) and 22% of the node positives (11/49) fail to express GPR30, while the remaining node positive and node negative cases maintain GPR30 expression. A similar trend was observed when comparing GPR30 expression to steroid hormone receptor status. Twenty eight percent of ER+ tumors (27/98) lacked GPR30. By comparison, 27% of ER- tumors (15/55) did not express GPR30. These data strongly indicate that GPR30 expression occurs independently of ER. Representative examples of ER+ and ER- tumors that either express or lack GPR30 are shown in figure 4.

Collectively these data are consistent with our hypothesis that GPR30 may serve as an independent marker for assessing antiestrogen responsiveness.

KEY RESEARCH ACCOMPLISHMENTS.

1. As a direct consequence of this funding instrument, we have generated immunohistochemical data regarding the expression of GPR30 in normal human tissue and in a significant number of breast cancer biopsy specimens (greater than 200). This data will lead to two manuscripts in the upcoming year. An additional manuscript has been submitted to the Journal of Cell Biology regarding the cell biological role of GPR30. Some of the data presented in these 3 manuscripts have been presented at three scientific symposia. Our results concerning the expression of GPR30 in human tissue and our studies concerning the biological role of GPR30 provide the basis for our ongoing collaboration with Procter and Gamble (see Reportable Outcomes below).

2. We have recently made use of tissue microarray (TMA) slides from the National Cancer Institute Cooperative Breast Cancer Tissue Resource (CBCTR). A single TMA slide contains as many as 200 archival specimens that have been generated from 0.6mm core samples of these tissues. TMAs are provided with histopathological diagnoses that have been provided by board- certified pathologists that work with the National Human Genome Research Institute. Other clinicopathological (tumor size and grade, node involvement, distant metastases, etc) and molecular markers (ie ER status, HER-2/neu expression) are provided with the TMAs in a double-blind fashion. We have had good success with utilizing our GPR30 peptide antibodies on TMAs and this tissue resource will greatly facilitate our study and expand the scope of our study.

3. Employing similar commercially available TMA composed of normal human tissues, we have expanded our survey of GPR30 expression. While this information is not directly outlined in our proposal, it provides information that is crucial to the assessment of GPR30 as a marker of estrogen-responsive tissue. We have assessed GPR30 expression in a wide array of normal tissues, including but not limited to: male and female reproductive tissues, skeletal bone, brain, hematopoietic tissues, tissues from the digestive and urinary system. The results of this survey are listed in Table I. Representative examples of GPR30-stained tissues are presented in 1 and 3 in the appendix.

4. We have analyzed 376 breast preinvasive and malignant breast carcinoma specimens that were collected on CBCTR TMAs for GPR30 expression. We have sorted GPR30 expression by tumor size, node involvement, and steroid hormone receptor status (ER/PR) and presented in Table III. This analysis extends our previous observations based on individual archival breast tumor biopsy specimens acquired from the Rhode Island Hospital pathology database (46 cases). Collectively, when these breast tumor samples are combined, we find no obvious correlation between any of the classic clinicopathological markers and GPR30 expression. This information is consistent with our hypothesis that GPR30 may serve as an independent marker for breast cancer. Further evaluation of samples from the CBCTR of patients receiving adjuvant therapies will help us to determine whether GPR30 has prognostic value in determining antiestrogen responsiveness.

REPORTABLE OUTCOMES.

1. Publications:

During the past year (third year of this award), we have generated important immunohistochemical data regarding the expression of GPR30 in normal human tissue and in a significant number of breast cancer biopsy specimens (greater than 200). This data will lead to two manuscripts in the upcoming year. We have submitted an additional manuscript to the Journal of Cell Biology regarding the cell biological role of GPR30.

Manuscripts:

Filardo, EJ, Quinn, JA, Frackelton, AR, Jr. and KI Bland (2002). Estrogen action via the G-protein-coupled receptor, GPR30: stimulation of adenylyl cyclase and cAMP-mediated attenuation of the EGFR-to-MAP K signaling axis. Molec Endocrinol. **16(1):** 70-84.

Filardo, E.J. (2002). Epidermal Growth Factor Receptor (EGFR) Transactivation by Estrogen via the G-Protein_Coupled receptor, GPR30: a Novel Signaling Pathway with Potential Significance for Breast Cancer. J. Steroid Biochem & Molec Biol. **80:** 231-238.

Quinn, JA, Graeber, CT, Calabresi, P, **Filardo, EJ** (2003) "Inside-out" integrin activation by the G-protein-coupled receptor, GPR30, promotes the EGF-like effects of estrogen. (Manuscript submitted to J Cell Biol)

Graeber, CT, Quinn, JA, Kim, D, Steinhoff, MM, Calabresi, P, **Filardo EJ** (2003) Estrogen receptor, ERα and GPR30, a heptahelical receptor that promotes the EGF-like effects of estrogen possess different tissue expression patterns. (manuscript in preparation).

Graeber, CT, Quinn, JA, Kim, D, Steinhoff, MM, Calabresi, P, Filardo EJ (2003) Expression of GPR30, a G-protein-coupled receptor that promotes the EGF-like effects of estrogen, in normal mammary epithelia and invasive mammary carcinoma. (manuscript in preparation).

Book chapter.

Filardo EJ, Quinn JA, and Graeber CT. (2002) Evidence supporting a role for GPR30, an orphan member of the G-protein–coupled receptor superfamily, in rapid estrogen signaling. In *Membrane-associated Steroid Hormone Receptors* pub by Kluwer Press Inc and edited by Cheryl S. Watson

2. Presentations. We were invited to present our work on GPR30 at:

Division of Cardiovascular Research, Proctor and Gamble Pharmaceuticals, "Expression of GPR30 in human tissues", October 8, 2002, Mason, OH

9th Annual T. J. Martell Cancer Consortium, "Regulation of the EGFR by estrogen in breast cancer cells", October 18-19, 2002, West Greenwich, RI

11th Annual International Congress on Hormonal Steroids and Hormones and Cancer in Fukuoka, Japan October 21-25, 2002

3. Research Award

We continue our work on our Research Scholar Award from the American Cancer Society (July 2002- June 2006) entitled "Estrogen Signaling via GPR30". With certainty, our ability to write and receive this ACS award was a direct result of the Career Development Award supported by the DOD. The ACS grant award will enable us to further investigate the mechanism by which GPR30 transactivates the EGFR. It is complementary in nature to the studies funded by the DOD to examine GPR30 expression in human breast biopsy specimens.

4. Invited Consultant on GPR30 action.

By invitation of Dr. Jan Rosenbaum, Ph.D., Principal Scientist, Cardiovascular Research, Procter & Gamble Pharmaceuticals, I continue to act as a consultant regarding a possible role of GPR30 in cardiovascular disease. One direct by-product of our interaction with PGP is that we have refined our immunohistochemical staining procedures using GPR30 peptide antibodies in archival breast biopsy specimens.

CONCLUSIONS.

The known estrogen receptors, ERα and ERβ, are the best prognostic indicators for determining responsiveness to antiestrogen therapy. Still, one in four patients with ER-positive tumors do not respond favorably to anti-estrogens, while one in six patients with ER-negative tumors exhibit objective tumor regression following antiestrogen therapy (Witliff, 1984). These clinical findings, in conjunction with data demonstrating that antiestrogens trigger rapid signaling events typically not associated with known ERs (Aronica et al, 1994; Lee et al, 2000; Filardo et al, 2000), raises the possibility that antiestrogens may, in part, exert their antitumor effects via non ER-dependent mechanisms.

It has long been suspected that other receptors, distinct from the ER, may participate in estrogen signaling. However, until recently the physical identity of these receptors has remained unknown. Within the past two years, we have provided data demonstrating that

the G-protein coupled receptor, GPR30, acts independently of known ERs to transmit intracellular signals that regulate the EGFR-to-MAP K signaling axis (Filardo et al, 2000; reviewed in Filardo, 2002; Filardo et al, 2001). This signaling axis holds particular significance for breast cancer in that it is frequently hyperactivated in breast cancer. Since antiestrogens also act as GPR30 agonists that regulate EGFR-to-MAP K signaling, the studies designed here will enable us to further determine whether there is a link between GPR30 expression, Erk hyperactivation and antiestrogen responsiveness.

From our examination of greater than 400 human breast tumor specimens, we have determined that there is no apparent correlation between ER status and GPR30 expression in human breast tumors (figure 3) and Table III. These data are consistent with our hypothesis that GPR30 may serve as an independent marker for assessing antiestrogen responsiveness.

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Wittliff JL (1984) Steroid-hormone receptors in breast cancer. Cancer **53:** 630- 643

Appendices.

A. Figures

Figure 1. Detection of GPR30 in normal human breast, thyroid, and hematopoietic tissues. Representative examples of archival, formalin-fixed biopsy specimens obtained from from normal human breast (A), lymph node (B), thyroid (C), or tonsil (D) that were immunostained with rabbit peptide antibodies raised against a C-terminal peptide from GPR30. Rabbit GPR30 C-terminal peptide antibodies were used at a 1: 2, 000 dilution and were visualized using biotinylated anti-rabbit immunoglobulin, avidin-conjugated horseradishperoxidase, and diaminobenzidine (brown) as substrate. A standard set of staining conditions were employed, however notice the differences in the degree of GPR30 reactivity in different normal human tissues. All samples were counterstained with hematoxylin (blue).

Figure 2. Detection of GPR30 in male reproductive tissues, kidney and pancreas.

Here, we show representative examples of normal prostate (A), seminal vesicle (B), kidney (C), or pancreas (D) with affinity-purified rabbit C-terminal GPR30 peptide antibodies. The tissue specimen has been counterstained with hematoxylin.

Figure 3. Detection of GPR30 in preinvasive breast cancer.

Archival breast biopsy specimens obtained from patients with preinvasive breast cancer were immunostained with GPR30 peptide antibodies. All specimens were counterstained with hematoxylin. Representative cases of: (A and D) atypical hyperplasia and (B) solid ductal carcinoma in situ (noncomedo) are shown. (C) is an adjacent serial section of (D) which has been stained with preimmune antibodies.

Figure 4. GPR30 expression does not correlate with estrogen receptor, ERα, expression in invasive breast cancer.

Unlike, normal mammary epithelia which appear uniformly positive for GPR30 scoring (+3) on a scale of 0 to +3 (as assessed from 6 individual normal mammoplasties), invasive ductal carcinoma of the breast exhibit range of GPR30 expression levels. Representative examples of archival, formalin-fixed breast tumor biopsy specimens obtained from patients with (A,B) ERα-positive (+) or (C, D) ERα-negative (-) invasive ductal carcinoma (IDC) are shown above. The ER(+) tumor in (A) scores as (+2) and the ER (-) tumor in (C) is (+3). The ER(+) and ER(-) tumors in (B) and (D) , respectively, are examples of GPR30-negative tumors.

B. Tables.

Table I. GPR30 expression in normal human tissues.

Table II. GPR30 expression in preinvasive breast cancer.

Table III. Correlation between GPR30 expression and clinicopathological markers in human breast tumor biopsy specimens.

C. Manuscripts.

Filardo EJ, Quinn JA, Frackelton AR Jr, Bland KI. Estrogen action via the G protein-coupled receptor, GPR30: stimulation of adenylyl cyclase and cAMP-mediated attenuation of the epidermal growth factor receptor-to-MAPK signaling axis. Mol Endocrinol. 2002 Jan;**16(1):**70-84.

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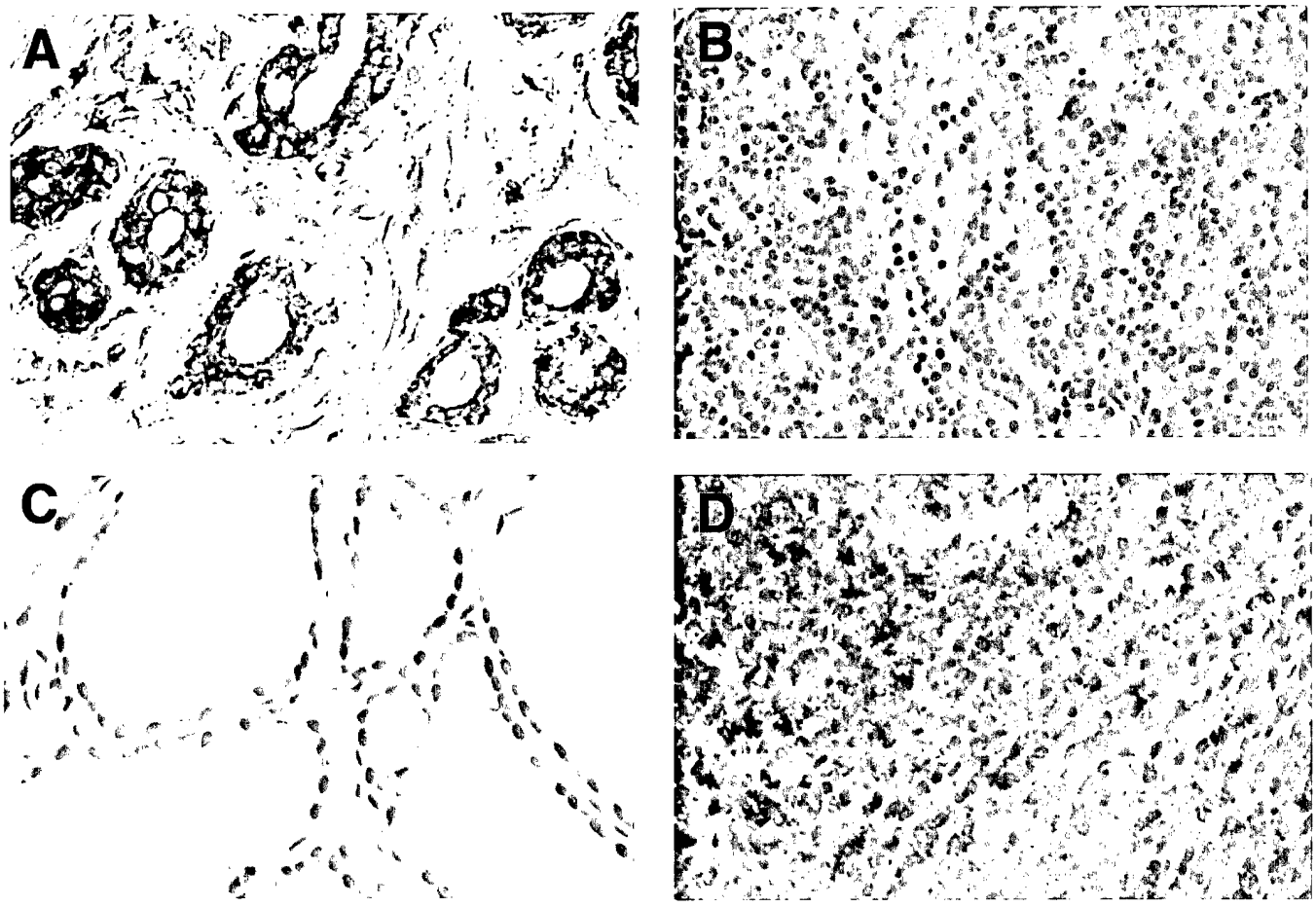

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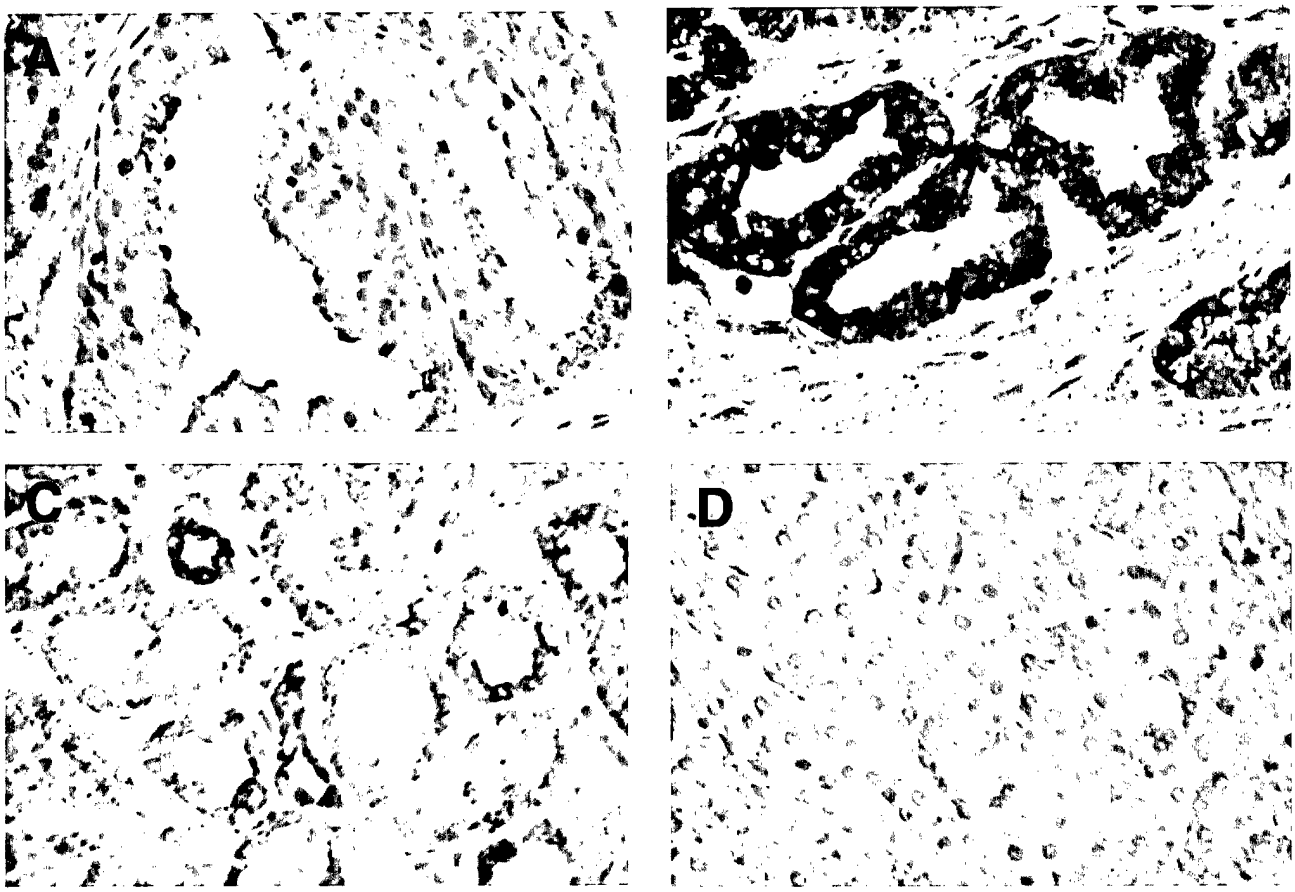

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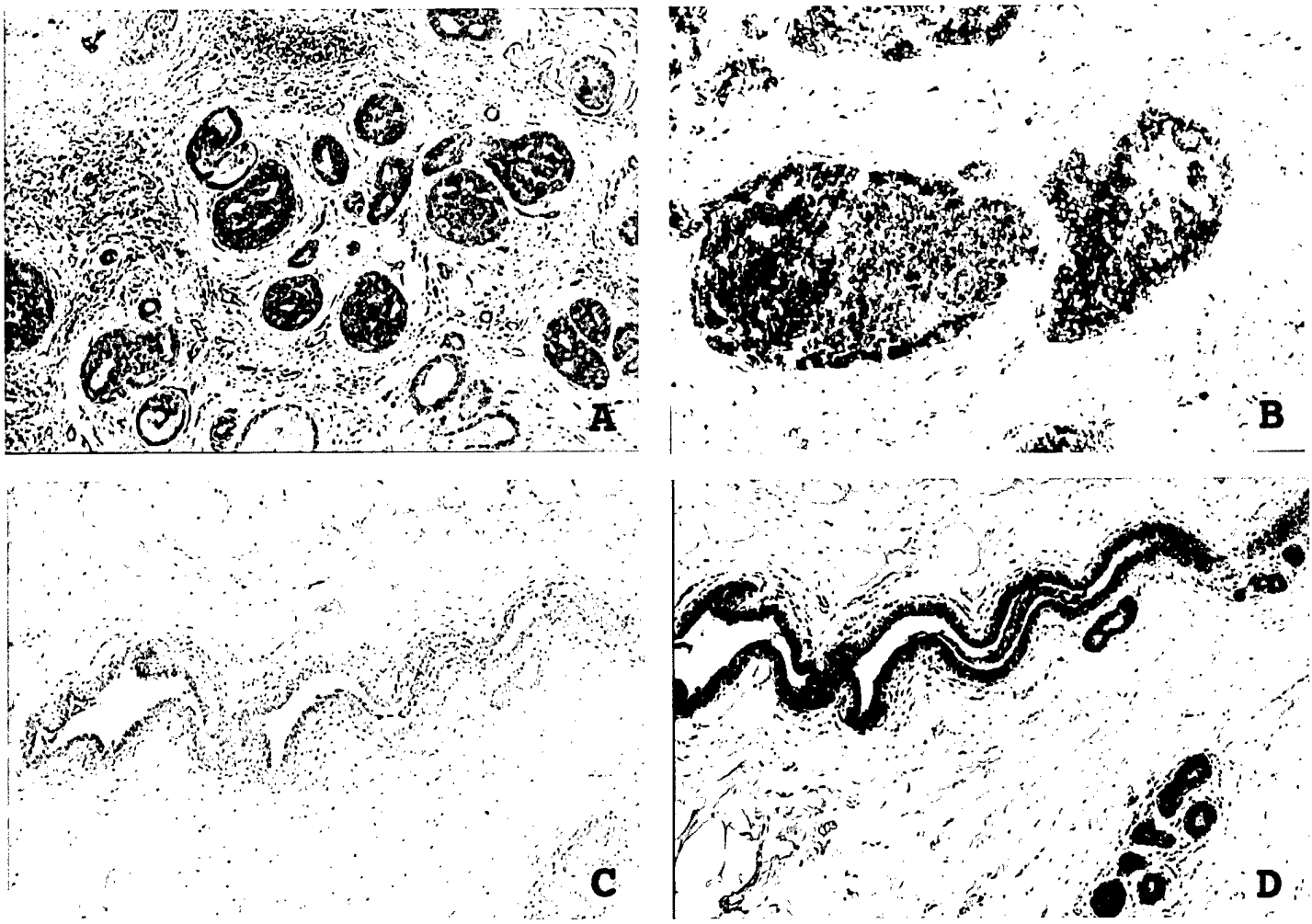

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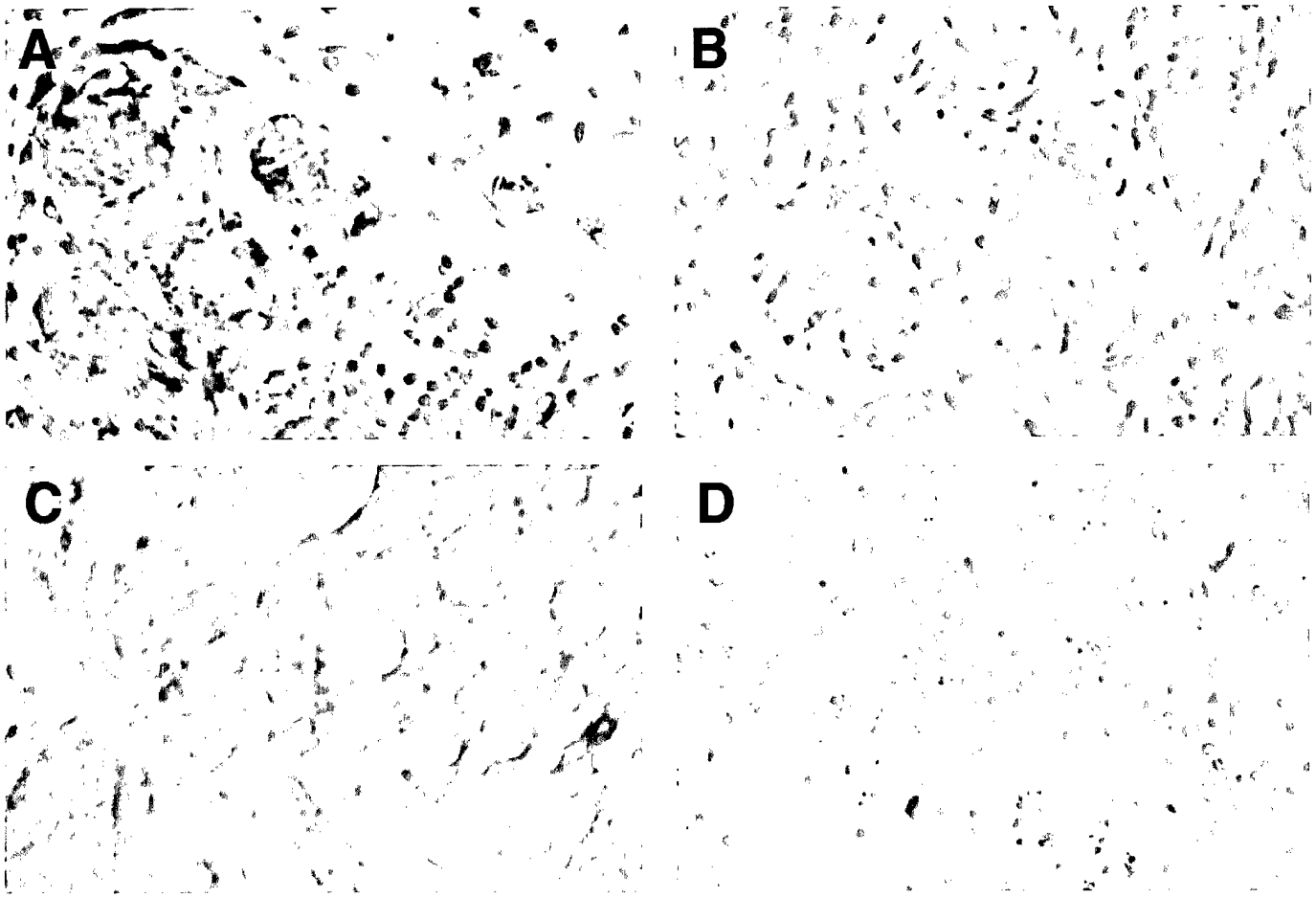

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Table I. GPR30 expression in normal human tissues.

Tissue type	*GPR30 expression*[a]
Reproductive tissues	
breast	+2
endometrium	+2
ovary	+2
placenta	+2
prostate	+2
seminal vesicle	+2
testis	+3
Hematopoietic tissues	
lymph node	0
spleen	0
tonsil	0
Other tissues	
adrenal gland	+2
kidney cortex	+2
pancreas	+2
appendix	0
cerebrum	0
heart	0
liver	0
skeletal muscle	0
thyroid	0

[a] GPR30 expression was scored on a scale ranging from 0 to +3.

Table II. GPR30 expression in preinvasive breast cancer.

Tissue type	*case*	*ER*[a]	*PR*[a]	*GPR30*[b]
benign hyperplasia	*1*	+	+	*+3*
apocrine metaplasia	*1*	+	-	*+2*
atypical ductal hyperplasia (ADH)	*1*	+	+	*+3*
	2	-	-	*+2*
ductal carcinoma in situ (DCIS)	*01-09m*	+	+	*0*
	02-09d	*nd*	*nd*	*0*
	03-03a	+	*nd*	*+1*
	04-04b	-	-	*+1*
	05-04c	+	+	*+1*
	06-08n	-	*nd*	*+1*
	07-15s	*nd*	*nd*	*+1*
	08-16s	+	+	*+1*
	09-16t	*nd*	*nd*	*+1*
	10-21h	+	+	*+1*
	11-03b	+	+	*+2*
	12-16r	+	+	*+2*
	13-20g	+	+	*+2*
	14-gp1	+	-	*+2*
	15-gp2	-	-	*+2*

[a] ER/PR status was provided by the National Cancer Institute in a blind fashion once GPR30 scores were reported.

[b] GPR30 expression was scored on a scale ranging from 0 to +3.

Table III. Correlation between GPR30 expression and clinico-pathological markers in human breast tumor biopsy specimens.

Tissue type	*GPR30 score*[a]				
	0	*+1*	*+2*	*+3*	*total*
Tumor size					
<2 cm	*23*	*26*	*21*	*5*	*75*
2-3 cm	*10*	*26*	*23*	*1*	*60*
3-4 cm	*6*	*10*	*6*	*1*	*23*
>4 cm	*1*	*2*	*0*	*0*	*3*
invasiveness					
node negative	*12*	*22*	*14*	*2*	*50*
node positive	*11*	*22*	*16*	*0*	*49*
distant metastases	*18*	*18*	*12*	*6*	*54*
hormone receptors					
ER+	*27*	*43*	*25*	*3*	*98*
ER-	*15*	*20*	*15*	*5*	*55*
PR+	*23*	*28*	*23*	*4*	*78*
PR-	*21*	*33*	*19*	*4*	*67*

[a] GPR30 expression was scored on a scale ranging from 0 to +3.

[b] ER/PR status, node involvement, and tumor size was provided by the National Cancer Institute

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Estrogen Action Via the G Protein-Coupled Receptor, GPR30: Stimulation of Adenylyl Cyclase and cAMP-Mediated Attenuation of the Epidermal Growth Factor Receptor-to-MAPK Signaling Axis

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Estrogen triggers rapid yet transient activation of the MAPKs, extracellular signal-regulated kinase (Erk)-1 and Erk-2. We have reported that this estrogen action requires the G protein-coupled receptor, GPR30, and occurs via Gβγ-subunit protein-dependent transactivation of the epidermal growth factor (EGF) receptor through the release of pro-heparan-bound EGF from the cell surface. Here we investigate the mechanism by which Erk-1/-2 activity is rapidly restored to basal levels after estrogen stimulation. Evidence is provided that attenuation of Erk-1/-2 activity by estrogen occurs via GPR30-dependent stimulation of adenylyl cyclase and cAMP-dependent signaling that results in Raf-1 inactivation. We show that 17β-E2 represses EGF-induced activation of the Raf-to-Erk pathway in human breast carcinoma cells that express GPR30, including MCF-7 and SKBR3 cells which express both or neither, ER, respectively. MDA-MB-231 cells, which express ERβ, but not ERα, and low levels of GPR30 protein, are unable to stimulate adenylyl cyclase or promote estrogen-mediated blockade of EGF-induced activation of Erk-1/-2. Pretreatment of MDA-MB-231 cells with cholera toxin, which ADP-ribosylates and activates Gαs subunit proteins, results in G protein-coupled receptor (GPCR)-independent adenylyl cyclase activity and suppression of EGF-induced Erk-1/-2 activity. Transfection of GPR30 into MDA-MB-231 cells restores their ability to stimulate adenylyl cyclase and attenuate EGF-induced activation of Erk-1/-2 by estrogen. Moreover, GPR30-dependent, cAMP-mediated attenuation of EGF-induced Erk-1/-2 activity was achieved by ER antagonists such as tamoxifen or ICI 182, 780; yet not by 17α-E2 or progesterone. Thus, our data delineate a novel mechanism, requiring GPR30 and estrogen, that acts to regulate Erk-1/-2 activity via an inhibitory signal mediated by cAMP. Coupled with our prior findings, these current data imply that estrogen balances Erk-1/-2 activity through a single GPCR via two distinct G protein-dependent signaling pathways that have opposing effects on the EGF receptor-to-MAPK pathway. (*Molecular Endocrinology* 16: 70–84, 2002)

EPIDERMAL GROWTH FACTOR (EGF) receptor (EGFR) belongs to a family of transmembrane tyrosine kinase receptors (EGFR/erbB1, HER2/erbB2, HER3/erbB3, and HER4/erbB4) that play a critical role in regulating normal cell growth and physiology (1). In general, EGFRs dictate cellular responses based on their ability to activate intracellular signaling cascades that effect biochemical events necessary to alter cell structure and function. The MAPKs, p42/44 MAPK [also known as extracellular signal-regulated kinase (Erk)-1/-2] are key downstream mediators of EGFR function because they phosphorylate and thereby modify the function of numerous proteins that collectively regulate polymerization of the actin cytoskeleton, mobilization of myosin, cell cycle checkpoints, and gene transcription (2).

Stimulation of the EGFR-to-MAPK pathway is initiated by the specific binding of cognate ligands, such as EGF, TGFα, heregulin, and heparan-bound EGF (HB-EGF), to specific EGFRs. This interaction results in the formation of EGFR homo- and heterodimers and autophosphorylation of tyrosyl residues within their cytoplasmic domains. Specific recognition of these phosphotyrosines by the adapter proteins Grb-2 and/or Shc, and guanine nucleotide exchange factors, such as Sos, serves to link-activated EGFR to MAPK via the monomeric GTPase, p21Ras. Thus activated, Ras is capable of recruiting the serine-threonine kinase Raf-1, which in turn promotes cascade phosphorylation and activation of Mek-1 and its dedicated substrates Erk-1 and Erk-2 (3). Under conditions of normal growth and behavior, activation of the EGFR to

Abbreviations: EGF, Epidermal growth factor; EGFR, EGF receptor; Erk, extracellular signal-regulated kinase; HB-EGF, heparan-bound EGF; GPCR, G protein-coupled receptor; ICI 182, 780, (7α-[9-[(4,4,5,5,5,-pentafluoropentyl)sulphinyl]nonyl]-estra-1,3,5(10)-triene-3,17β-diol), a high affinity ER antagonist; MBP, myelin basic protein; Mek, MAPK/ERK kinase (same as MAP kinase kinase); SHBGR, a membrane receptor on breast and prostate cancer cells.

MAPK pathway is transient and attenuated by a variety of control mechanisms, which prevent downstream activation of Erk-1/-2 (4), as well as by phosphatases, which dephosphorylate, and thereby inactivate, Erk-1/-2 (5). In contrast, constitutive activation of the EGFR to MAPK pathway results in dysregulated cellular behaviors associated with carcinogenesis (6, 7).

Several lines of evidence suggest that dysregulation of the EGFR to MAPK pathway may have particular significance for breast carcinogenesis. First, overexpression of the EGFR family member, HER2, is a common event in breast tumors (8), an event that is known to increase both the amplitude and duration of EGF-stimulated Erk-1/-2 activation (9). Second, Erk-1-mediated phosphorylation of serine residue 118 of the ER enhances its gene activation function (10, 11). Third, estrogen stimulates activation of Erk-1/-2 (12–15). In this regard, constitutive Erk-1/-2 may initiate dysregulated cellular behaviors exhibited by estrogen-independent tumors; additionally, Erk-1/-2 may also provide a mechanism whereby hyperactive growth factor signaling may activate estrogen-dependent tumor growth. The association of increased Erk-1/-2 activity with invasive breast cancer suggests this hypothesis (16).

Aside from receptor tyrosine kinases, as represented by the EGFRs, G protein-coupled receptors (GPCRs) comprise a second major class of transmembrane receptors that signal via Erk-1/-2. Unlike EGFRs, GPCRs activate Erk-1/-2 through several distinct mechanisms, some of which couple via the monomeric GTPases, Ras or Rap; others activate Raf or Mek directly (17). In some instances, GPCR stimulation leads to the activation of Src-related tyrosine kinases and the assembly of Grb-2/Sos/Shc complexes on the cytoplasmic domain of EGFRs (18). In conjunction with the finding that Src can directly phosphorylate the EGFR (19), these observations suggest that GPCRs may activate EGFRs via Src-mediated phosphorylation of the EGFR cytoplasmic tail. More recently, ligands for some GPCRs, including endothelin, bombesin, and lysophosphatidic acid have been shown to transactivate the EGFR through their ability to cleave and release surface-associated precursors of EGF-related polypeptides (20). These findings parallel observations that other receptors that lack intrinsic enzymatic function, such as integrins (21) and cytokine receptors (22), also transactivate the EGFR. The fact that may different receptors transactivate the EGFR to MAPK pathway suggests that coordinated signaling is required to regulate the activity of this commonly used signaling axis.

Recently, we have shown that GPR30 is required for estrogen-induced activation of the MAPKs, Erk-1 and Erk-2 (23). This activation response is rapid and occurs via $G\beta\gamma$-subunit protein-dependent release of surface-associated HB-EGF and transactivation of the EGF receptor. GPR30-dependent, estrogen-mediated Erk-1/-2 activation is transient, rapidly returning to basal levels 10–15 min after initial exposure to estrogen. This rapid inactivation of Erk-1/-2 implies the existence of a tightly controlled regulatory mechanism. Others have shown that estrogen (24–26) also promotes stimulation of adenylyl cyclase activity and production of intracellular cAMP. In some cell settings, cAMP acts as a potent inhibitor of Erk-1/-2 activity (27, 28). In other cell types, Erk-1/-2 are activated by cAMP via its ability to promote B-raf-mediated stimulation of Mek-1 (29). Because adenylyl cyclases are commonly linked to GPCRs (30, 31) we investigated whether GPR30 participates in estrogen-mediated stimulation of adenylyl cyclase. Here, we show that GPR30 is required for estrogen-induced stimulation of adenylyl cyclase and cAMP-mediated inhibition of Erk-1/-2. Moreover, we demonstrate that ER antagonists, including the antiestrogens tamoxifen and 7α-[9-[(4,4,5,5,5,-pentafluoropentyl)sulphinyl]nonyl]estra-1,3,5(10)-triene-3,17β-diol (ICI 182,780), can also induce these same GPR30-dependent rapid signaling events. Our results suggest that estrogens and antiestrogens signal via GPR30-mediated stimulation of adenylyl cyclase to inhibit the EGFR to MAPK pathway.

RESULTS

Estrogen-Mediated Stimulation of Adenylyl Cyclase Activity Is ER-Independent and Requires the Expression of GPR30

Estrogen stimulates intracellular cAMP production through its ability to activate adenylyl cyclase in the plasma membrane via an as-yet-to-be determined mechanism (24, 25). Prior studies demonstrating estrogen promotes this activity in MCF-7 cells that express known estrogen receptors has led to the hypothesis that the ER may regulate adenylyl cyclase activity (24). More recent data have shown that $G\alpha s$-proteins are required in these cells for estrogen-mediated stimulation of adenylyl cyclase (32). Traditionally, adenylyl cyclase activity is known to be regulated by receptors that couple to heterotrimeric G proteins (30). Although the ER has been shown to exist in the plasma membrane (33, 34), there are no known functional motifs within the structure of the ER that permit $G\alpha s$ protein coupling or activation (35). Because we have shown that GPR30 is required for transactivation of the EGFR by estrogen (23), we queried whether this receptor, or the known ERs, promote estrogen-mediated stimulation of adenylyl cyclase.

To discriminate between these possibilities, we measured the ability of estrogen to stimulate cAMP production in membranes isolated from human SKBR3 breast cancer cells that express neither ERα nor ERβ (36) yet express GPR30 protein (23). SKBR3 membranes exposed to 17β-E2 produced substantial levels of cAMP (Fig. 1A). This activity was not promoted by the isomer, 17α-E2. In agreement with the observations of Aronica and colleagues (24), demonstrating that ER antagonists can stimulate adenylyl cyclase activity in MCF-7 membranes, the antiestrogen ICI 182, 780, also stimulated cAMP production in

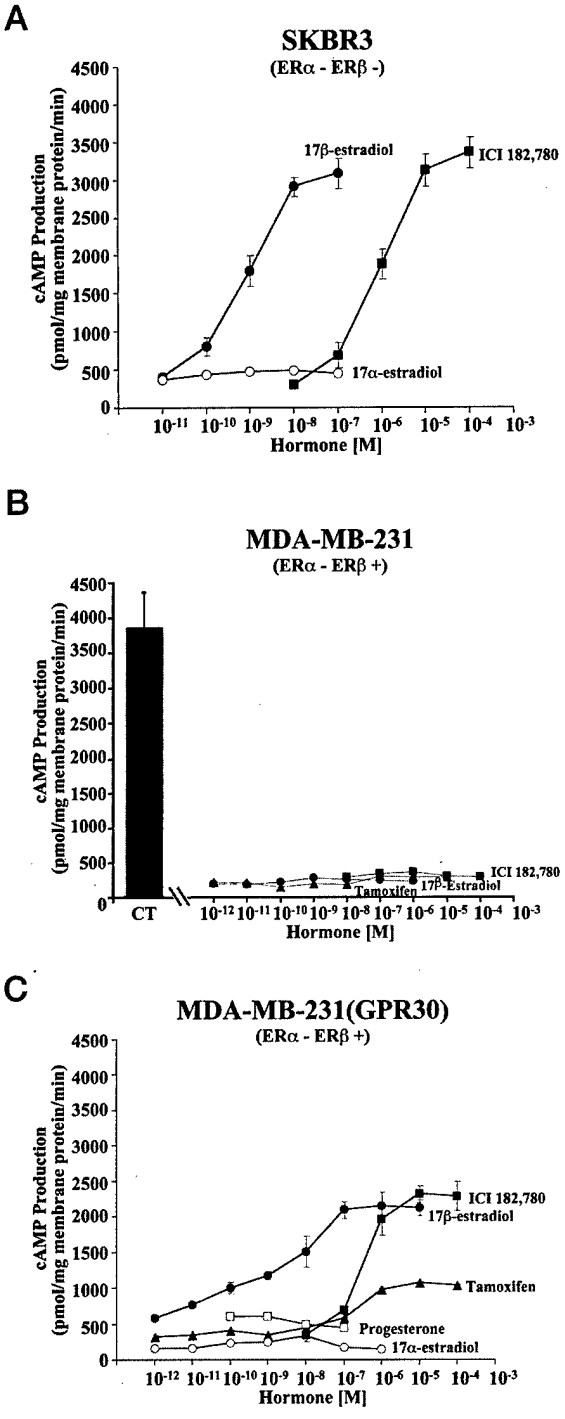

Fig. 1. Estrogen Stimulation of Adenylyl Cyclase Activity Is ER Independent and Requires the Expression of GPR30

Adenylyl cyclase activity was determined from membranes prepared from SKBR3 as well as vector- or GPR30-transfected MDA-MB-231 human breast cancer cells which were stimulated with either cholera toxin (CT) (1 μg/ml) or various concentrations of 17β-E2, 17α-E2, 4-hydroxy-tamoxifen, ICI 182, 780, or progesterone. The y-axis values are on a linear scale and represent picomoles of cAMP generated per milligram of membrane protein per minute. The x-axis values are expressed on a logarithmic scale as the molar concentration of hormone. Each data point represents the mean ± the SD of quadruplicate samples.

membranes from SKBR3 cells (Fig. 1A). In contrast, as had been previously noted by others (24), we found that membranes from MDA-MB-231 cells that express ERβ but not ERα protein (37) did not generate cAMP upon exposure to either 17β-E2 or ER antagonists (Fig. 1B). Nevertheless, cholera toxin, an agonist that ADP-ribosylates and directly activates Gαs subunit proteins, stimulated a 15-fold increase in cAMP in MDA-MB-231 membranes, indicating that the MDA-MB-231 membrane preparations retained Gαs proteins capable of activating adenylyl cyclase (Fig. 1B).

Membranes prepared from MDA-MB-231 cells that were forced to overexpress GPR30 protein were tested for their ability to produce cAMP in response to estrogen stimulation. We found that membranes isolated from GPR30-transfected MDA-MB-231 cells supported stimulation of adenylyl cyclase after exposure to either 17β-E2, tamoxifen, or ICI 182, 780 (Fig. 1C). The saturation dose for 17β-E2-mediated stimulation of adenylyl cyclase activity was near 1 μM, whereas approximately 10 nM of 17β-E2 showed a half-maximal response. Half-maximal stimulation was achieved with 0.2 μM ICI 182, 780, a concentration that closely approximates the half-maximal dose for a response of similar amplitude in SKBR3 cells (Fig. 1A). No increases in cAMP production were observed in MDA-MB-231 (GPR30) membranes treated with 17α-E2, an isomer of 17β-E2 that is unable to support ER function. Similarly, the sex steroid hormone progesterone failed to elicit cAMP production from MDA-MB-231 (GPR30) membranes. Collectively, these results indicate that GPR30 acts independently of the known ERs to promote estrogen-mediated stimulation of adenylyl cyclase.

Inhibition of PKA Prolongs Estrogen-Induced Erk-1 and Erk-2 Activity

Agents that elevate intracellular cAMP possess either stimulate or inhibit Erk-1/-2 activity in different cell types (27–29). The ability of cAMP to activate Erk-1/-2 has been attributed to the cellular expression of the 95-kDa isoforms of B-Raf (29). Therefore, we measured B-Raf expression in MDA-MB-231 (GPR30) cells by Western blotting (Fig. 2A). Simian SV40-transformed COS-7 kidney epithelial cells, which undergo Erk-1/-2 activation in response to cAMP, expressed elevated levels of both the 95-kDa and 68-kDa isoforms of B-Raf. In contrast, MDA-MB-231 (GPR30) breast cancer cells grown in serum expressed the 95-kDa isoform of B-Raf, and little, if any, detectable 68-kDa B-Raf (Fig. 2A). However, we found that when these cells were serum-starved they failed to express detectable levels of 95-kDa B-Raf. This finding is consistent with a prior report noting that the 95-kDa isoform of B-Raf is inhibited in serum-starved cells (38) and suggests that serum-starved MDA-MB-231(GPR30) cells may be refractory to cAMP-dependent activation of Erk. To directly test this hypothesis, Erk-1/-2 phosphorylation was measured after exposure of MDA-MB-231 (GPR30) cells to either cholera toxin or dibutyryl cAMP, a

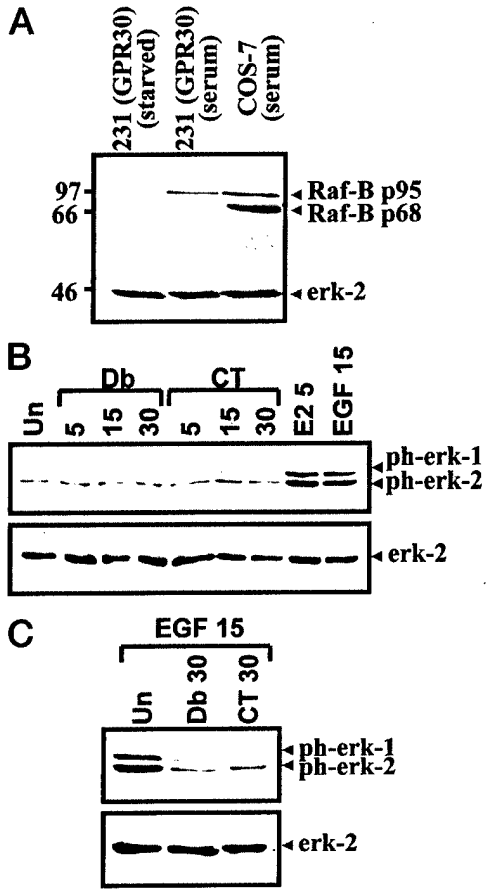

Fig. 2. cAMP Does Not Activate Erk-1/-2 in MDA-MB-231 (GPR30) Breast Cancer Cells

A, Fifty micrograms of protein from whole-cell lysates of COS-7 cells or MDA-MB-231 (GPR30) cells grown in serum, or starved, were subjected to Western blotting with a B-Raf-specific antibody that recognizes both the 95- and 68-kDa isoforms. The same filter was reprobed with Erk-2 antibodies to confirm equivalent protein loading. B, Serum-starved MDA-MB-231(GPR30) cells were untreated or treated with cholera toxin (1 μg/ml), dibutyrl cAMP (1 mM), 17β-E2 (1 nM), or EGF (1 ng/ml) for various lengths of time (minutes) and lysed in detergent. C, Alternatively, cells were preexposed to cholera toxin (1 μg/ml), dibutyrl cAMP (1 mM), or left untreated for 30 min and then stimulated with EGF (100 ng/ml) for 15 min prior to lysis. Fifty micrograms of protein from each detergent lysate was electrophoresed through 15% reducing SDS-polyacrylamide gels, transferred to nitrocellulose, and probed with antibodies specific for phosphorylated Erk-1 and -2. The nitrocellulose membrane was then stripped and reprobed with antibodies that recognize total (phosphorylation state-independent) Erk-2 protein. The position of phosphorylated Erk-1/-2 protein or total Erk-2 protein are indicated at *left*.

membrane-permeable cAMP congener. Although either estrogen or EGF induced Erk-1/-2 activation in MDA-MB-231 (GPR30) cells, neither cholera toxin nor dibutyryl cAMP promoted Erk-1/-2 stimulation (Fig. 2B). However, MDA-MB-231 (GPR30) cells exposed to either cholera toxin or dibutyrl cAMP were able to blunt EGF-induced Erk-1/-2 activity, suggesting that cAMP antagonizes Erk in these cells (Fig. 2C). This finding implies that via its ability to stimulate adenylyl cyclase, estrogen may transmit a cAMP inhibitory signal that acts to attenuate estrogen-mediated, transactivation of the EGFR-to-Erk signaling axis.

To investigate whether the restoration of Erk-1/-2 from peak activity levels to basal levels in MDA-MB-231 (GPR30) cells was associated with 17β-E2-induced cAMP-dependent inhibition of Erk-1/-2, we determined the effects of KT5720, a cAMP-dependent PKA inhibitor on the kinetics of estrogen-mediated Erk-1/-2 activation. After stimulation, detergent lysates were prepared and Erk-1/-2 activity and expression was determined by immunoblotting using phosphorylation state-dependent and -independent antibodies. As previously reported (23), 17β-E2-induced a rapid increase in the phosphorylation state of Erk-1 and Erk-2 in these cells. However, as observed in Fig. 3, the duration of this response is transient. Increases in Erk-1/-2 phosphorylation were detected as early as 1 min after exposure to 17β-E2. Peak Erk-1/-2 phosphorylation levels occurred at 5 min (3- to 4-fold increase) with Erk-1/-2 activity returning to baseline levels by 30–60 min. Cells exposed to KT5720 for 2 h exhibited reduced basal levels of Erk-1/-2 activity relative to untreated control cells. However, after estrogen stimulation, the rate and amplitude of the Erk-1/-2 activation response in KT5720 pretreated cells was similar to that observed in control cells with peak activity observed within 5 min. In contrast to untreated control cells, KT5720-treated cells maintained elevated levels of Erk-1/-2 activity for an extended period of time (greater than 1 h) after estrogen stimulation (Fig. 3). This observation suggests that activation of cAMP-dependent PKA is required to restore estrogen-induced Erk-1/-2 activity to basal levels.

Estrogen Represses EGF-Induced Erk-1/-2 Activation Via Its Ability to Generate cAMP Via GPR30

To further assess the mechanism by which estrogen inhibits Erk-1/-2 activity, we examined the ability of 17β-E2 to suppress EGF-induced Erk-1/-2 phosphorylation. As described previously (23) and shown in Fig. 4A, stimulation of quiescent MCF-7 cells (ERα+, ERβ+, GPR30+) with EGF induces substantial (5- to 10-fold) increases in the phosphorylation state, or activity, of Erk-1/-2 within 15 min. Pretreatment of MCF-7 cells with 17β-E2 for 30 min significantly inhibited EGF-induced Erk-1/-2 phosphorylation or activity (Fig. 4A). This state of E2-induced suppression of EGF-induced Erk-1/-2 phosphorylation could be measured in cells maintained in 17β-E2 for as long as 120 min prior to EGF stimulation. Reprobing these filters with phosphorylation state-independent Erk-2 antibodies verified that these changes in Erk-1/-2 phosphorylation were not due to changes in Erk-2 protein expression. To address whether the suppressive effect of estrogen on EGF-stimulated Erk-1/-2 activity might be due to a delay of the onset of EGF-induced

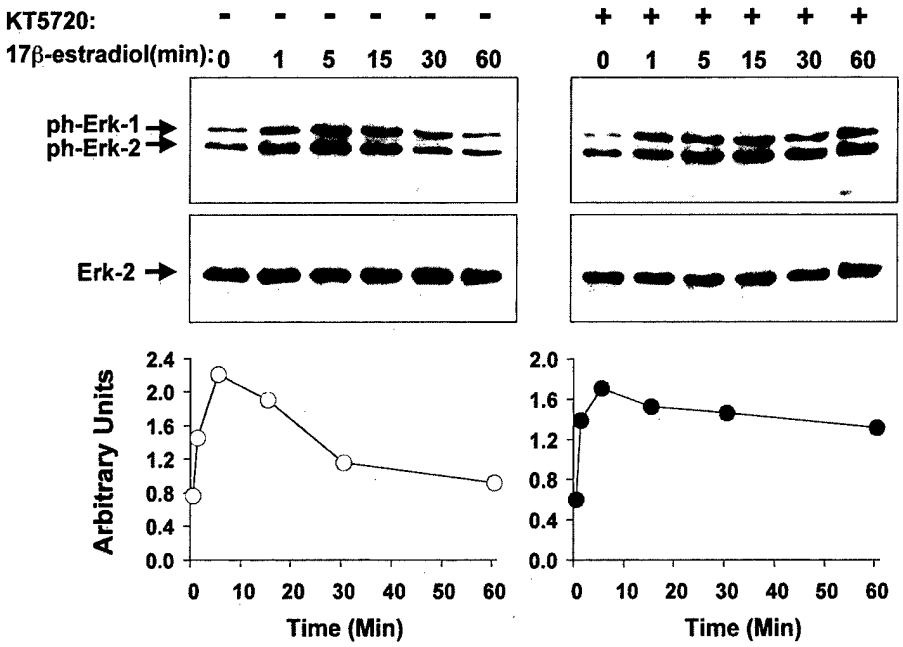

Fig. 3. Inhibition of PKA Activation Results in Prolonged Estrogen-Mediated Activation of Erk-1/-2

Serum-deprived human MCF-7 breast adenocarcinoma cells were pretreated with the cAMP congener, KT5270, or vehicle before stimulation with 1 nM 17β-E2 (17β-E2) for the indicated lengths of time (minutes) and then lysed in detergent. Expression of phosphorylated Erk-1/-2 or total Erk-2 protein was determined as described in Fig. 2. The position of phosphorylated Erk-1/-2 protein or total Erk-2 protein are indicated at *left*. The data shown are representative of at least three independent experiments. *Below,* Band intensities from this experiment were quantified using NIH Image software. Results were normalized to total Erk-2 expression in each sample and plotted as arbitrary units.

Erk-1/-2 activation, Erk-1/-2 phosphorylation was measured in MCF-7 cells that were pretreated with estrogen and then stimulated with EGF for various lengths of time. Basal Erk-1/-2 phosphorylation levels were observed in cells that had been pretreated with 17β-E2 and subsequently challenged with EGF for any of the time intervals tested (Fig. 4B), indicating that 17β-E2 did not delay the onset of EGF-induced Erk-1/-2 activity in these cells. ER-antagonists, 4-hydroxytamoxifen or ICI 182, 780 behaved similarly to 17β-E2 with regards to their ability to attenuate EGF-induced Erk-1/-2 phosphorylation (data not shown). To determine whether the estrogen-induced suppressive effect on EGF-induced Erk-1/-2 activity also occurs via activation of cAMP-dependent PKA, MCF-7 cells were incubated with KT5720 before exposure to tamoxifen and then stimulated with EGF. KT5720-treatment completely abrogated tamoxifen-mediated attenuation of EGF-induced phosphorylation of Erk-1/-2 in these cells (Fig. 5A). No changes were observed in the expression of total Erk-2 protein in response to KT5720, whereas this treatment abolished tamoxifen-mediated repression of EGF-induced Erk-1/-2 activity (Fig. 5A). We found that estrogen suppression of EGF-induced Erk-1/-2 was also observed in ER-negative SKBR3 cells (Fig. 5B). Repression of EGF-induced Erk-1/-2 activity in these cells was achieved by not only 17β-E2 but also the ER-antagonists tamoxifen and ICI 182, 780 (Fig. 5B). As was the case for MCF-7 cells (Fig. 5A), estrogen-mediated repression of EGF-induced Erk-1/-2 activation in SKBR3 cells was similarly sensitive to the cAMP congener, KT5720 (Fig. 5B). Because KT5720 functions as an inhibitor of cAMP-dependent PKA (39), our data suggests that PKA-mediated, cAMP-dependent signaling is necessary for repression of Erk-1/2 activity by estrogens and antiestrogens. These findings indicate that the ER is not required for this estrogen suppressor activity.

To determine whether GPR30 might promote this estrogen suppressor activity, we compared the effect of estrogen on EGF-induced stimulation of Erk-1/-2 activity in parental MDA-MB-231 or MDA-MB-231 cells forced to overexpress GPR30 protein. Upon exposure to EGF, serum-deprived MDA-MB-231 cells exhibited a 3- to 5-fold increase in Erk-1/-2 phosphorylation and activity (Fig. 6A). Prior exposure to tamoxifen (Fig. 5A) or 17β-E2 (data not shown) did not inhibit EGF-induced stimulation of Erk-1/-2 phosphorylation in these cells. However, exposure of these parental MDA-MB-231 cells to either dibutyrl cAMP or the potent cAMP agonist, cholera toxin resulted in a dramatic reduction of EGF-stimulated Erk-1/-2 activity and phosphorylation (Fig. 6A). In contrast, GPR30-transfected MDA-MB-231 cells expressed the estrogen suppressor phenotype. These cells exhibited 20-fold less EGF-induced Erk-1/-2 phosphorylation after tamoxifen treatment than mock-transfected MDA-MB-

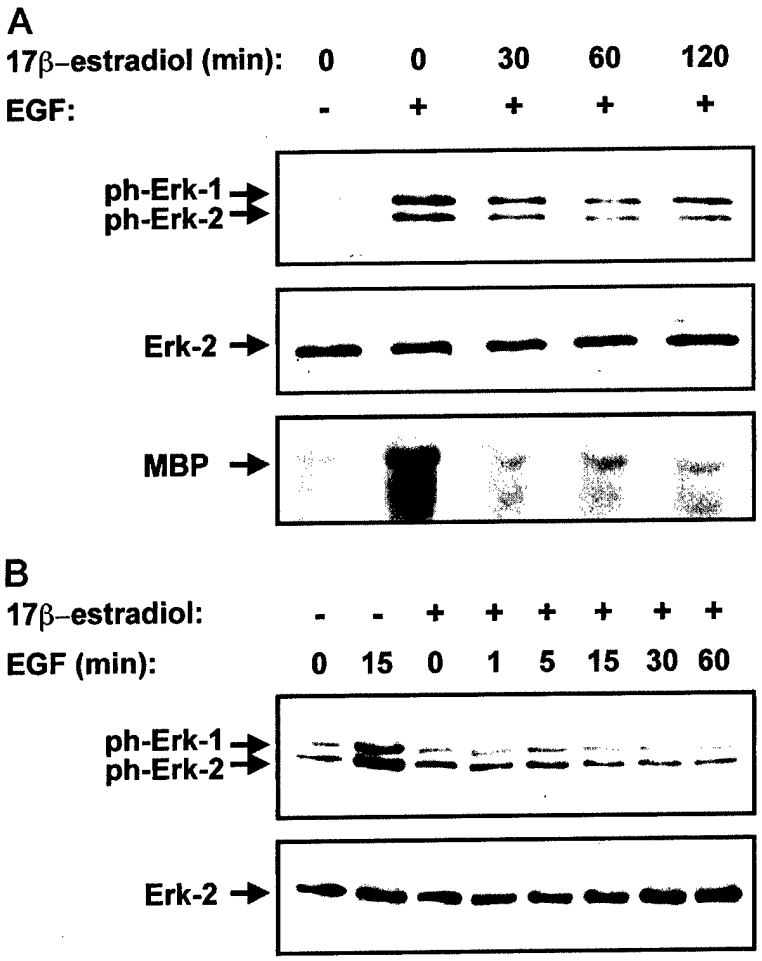

Fig. 4. Attenuation of EGF-Induced Erk-1/-2 Activity by Estrogen

Phospho-Erk expression was determined in serum-deprived MCF-7 cells that were exposed to estrogen before EGF-stimulation. A, Cells were pretreated with 1 nM 17β-E2 for various lengths of time (0–120 min) and then stimulated with 100 ng/ml EGF for 15 min and lysed in detergent. B, Cells were pretreated with 1 nM 17β-E2 for 30 min and then stimulated with 100 ng/ml of EGF for various lengths of time (1–60 min) and then extracted in detergent. Expression of phosphorylated Erk-1/-2 or total Erk-2 protein was determined as described above.

231 cells (Fig. 7). A similar inhibition of EGF-induced Erk-1/-2 phosphorylation was observed for MDA-MB-231(GPR30) cells treated with 17β-E2 (data not shown). However, attenuation of EGF-induced Erk-1/-2 phosphorylation was not inhibited in MDA-MB-231 (GPR30) cells exposed to 500 nM of either the inactive 17α-E2 isomer or progesterone (Fig. 8). No differences were observed between vector- and GPR30-transfected MDA-MB-231 cells in total Erk-2 protein expression under any of these conditions (Figs. 7 and 8). Thus, collectively these data suggest that the cAMP-signaling pathway promoting estrogen-mediated repression of Erk-1/-2 is intact in MDA-MB-231 cells, and that these cells are unable to potentiate estrogen suppressor activity due to a defect in the pathway leading to Gαs-subunit protein activation. Overexpression of GPR30 protein reconstitutes the estrogen suppressor phenotype suggesting that GPR30 is required for estrogen-mediated suppression of the EGFR-to-MAPK signaling axis. Moreover, these data provide specificity for the GPR30-dependent responses measured here, and suggest a novel mechanism by which estrogenic hormones can regulate growth factor signaling.

Attenuation of Estrogen-Induced Erk-1 and Erk-2 Activity Does Not Effect EGFR Activation or Internalization

We have previously demonstrated that estrogen stimulation of GPR30-expressing breast carcinoma cells results in transactivation of the EGFR through release of surface-associated HB-EGF (23). To determine whether attenuation of estrogen-induced Erk-1/-2 activity is associated with a decrease in EGFR activity, EGFR tyrosine phosphorylation was measured in detergent lysates prepared from MDA-MB-231(GPR30) cells that were exposed to estrogen for various peri-

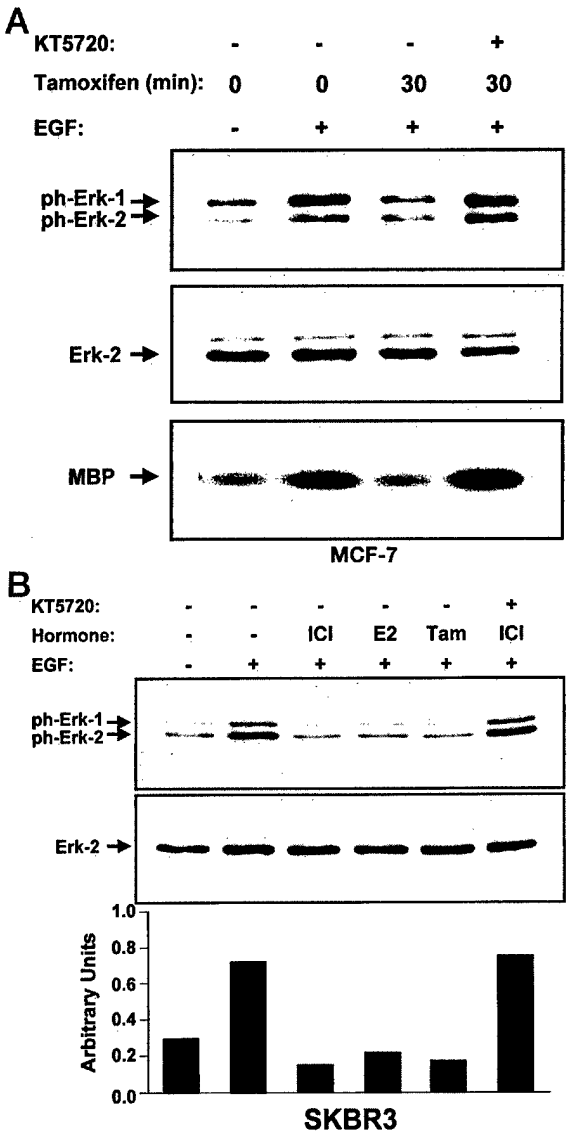

Fig. 5. Attenuation of EGF-Induced Erk-1/-2 Activity by Estrogens or Antiestrogens Is Abrogated by the cAMP Congener, KT5720

After a 1-h exposure to KT5720 (10 μM) or vehicle (DMSO), MCF-7 cells (A) or SKBR3 cells (B) were treated with 17β-E2 (1 nM), 4-hydroxy-tamoxifen (1 μM), or ICI 182, 780 (1 μM) for 30 min and then stimulated with EGF (100 ng/ml; 15 min). Detergent extracts were prepared and the expression of phosphorylated Erk-1/-2 or total Erk-2 protein were determined as described previously. *Below,* Band intensities from this experiment were quantified using NIH Image software. Results were normalized to total Erk-2 expression in each sample and plotted as arbitrary units.

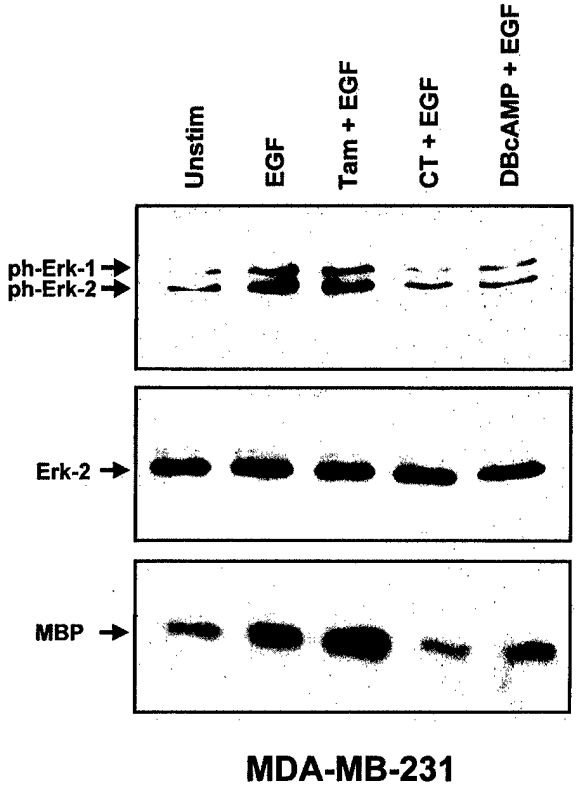

Fig. 6. Tamoxifen-Mediated Attenuation of EGF-Induced Phosphorylation of Erk-1/-2 Does Not Occur in MDA-MB-231 Breast Carcinoma Cells

MDA-MB-231 (ERα− ERβ+) breast carcinoma cells were pretreated with either 1 μm 4-hydroxytamoxifen (Tam), 1 μg/ml cholera toxin (CT) or 1 mM dibutyrl cAMP (dB) for 1 h, stimulated with 100 ng/ml EGF for 15 min, and detergent lysates were prepared. Expression of phospho-Erk-1/-2 and total Erk-2 protein was determined as previously described. Erk-1/-2 activity was measured from these lysates by standard immune complex kinase assay using MBP as an exogenous substrate.

ods of time. Significant EGFR tyrosine phosphorylation was observed as early as 3 min following exposure to 17β-E2 (Fig. 9A). Comparable amounts of tyrosine phosphorylated erbB1/EGFR was observed at 60 min after estrogen stimulation (Fig. 9A), even though basal levels of phosphorylated Erk-1/-2 are present at these later time points (Fig. 3). To further investigate whether restoration of Erk-1/-2 to basal levels of activity after estrogen stimulation may be the consequence of EGF receptor down-modulation, surface expression of erbB1/EGFR was measured after estrogen stimulation (Fig. 9B). MDA-MB-231(GPR30) cells were treated with 17β-E2 or EGF, or pretreated with 17β-E2 for 30 min and then exposed to EGF. After stimulation at 37 C, cells were fixed in paraformaldehyde, immunostained with Ab-1, an ErbB1/EGFR-specific monoclonal antibody directed against an epitope that maps outside the EGF-binding pocket of the receptor, and analyzed by flow cytometry. As observed in Fig. 9B, exposure of cells to EGF (100 ng/ml) for 15 min resulted in a 50% decrease in surface EGFR. In contrast, less than a 5% decrease in surface EGFR was observed in cells exposed to estrogen for 3, 10, 30, or 60 min. Yet, cells which were pre-exposed to 17β-E2, internalized 50% of their surface EGFR within 15 min subsequent to stimulation with EGF (Fig. 9B). Taken together, these data imply that the restoration

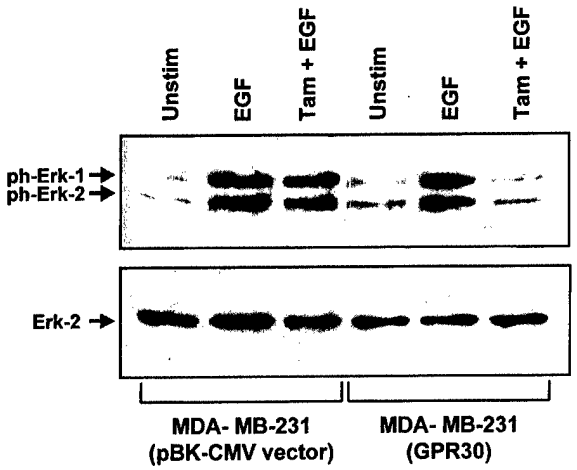

Fig. 7. Expression of GPR30 in MDA-MB-231 Breast Carcinoma Cells Restores Estrogen-Mediated Repression of EGF-Induced Erk-1/-2 Phosphorylation

Detergent lysates were prepared from vector- or GPR30-transfected MDA-MB-231 cells that were unstimulated, EGF stimulated, or pretreated with 1 μM 4-hydroxytamoxifen (Tam) for 1 h before EGF stimulation. Fifty micrograms of cellular protein was electrophoresed through SDS-polyacrylamide and transferred to nitrocellulose. Phosphorylated Erk-1/-2 proteins were detected by immunoblotting with phospho-Erk-specific antibodies. The membrane was then stripped and reblotted with antibodies that detect total Erk-2 protein.

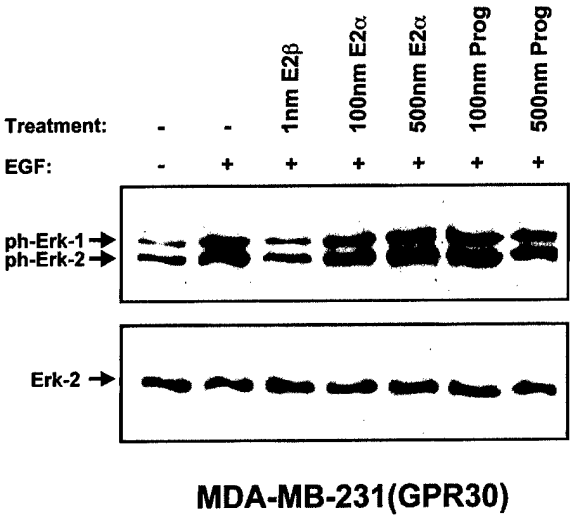

Fig. 8. GPR30-Dependent Attenuation of EGF-Induced Activation of Erk-1/-2 Does Not Occur in Cells Treated with 17α-E2 or Progesterone

Expression of phospho-Erk or total Erk-2 protein was measured in detergent lysates prepared from GPR30-transfected MDA-MB-231 breast carcinoma cells that were pretreated with either 17β-E2 (E2β), 17α-E2 (E2α), or progesterone (Prog) for 1 h prior to stimulation with 100 ng/ml EGF for 15 min.

of estrogen-induced Erk-1/-2 activity to basal levels observed by 30 min following exposure to estrogen is not due to a decrease in EGFR activity or expression and suggests that the estrogen-induced blockade of Erk-1/-2 activity occurs downstream of the EGFR.

Estrogen-Mediated Attenuation of EGF-Induced Erk-1 and Erk-2 Activity Is the Result of Raf-1 Inactivation

To better define the mechanism associated with estrogen-mediated repression of EGF-induced Erk-1/-2 activation, we measured the phosphorylation status of Mek-1 and the activity of Raf-1, which serve as intermediate components of the EGFR-to-Erk cascade. EGF stimulation of MDA-MB-231(GPR30) cells induced rapid Mek-1 phosphorylation (Fig. 9C) and Raf-1 activity (Fig. 9D). 17β-E2 stimulation of these cells also induced rapid, yet transient, Mek-1 and Raf-1 phosphorylation and activity with a kinetic response that paralleled the activation response observed for estrogen-induced Erk-1/-2 phosphorylation observed in Fig. 3. Both Raf-1 and Mek-1 activation by 17β-E2 in this cell background is dependent on GPR30 expression (data not shown). Pretreatment with 17β-E2 abrogated both EGF-induced Mek-1 phosphorylation (Fig. 9C) and Raf-1 activation (Fig. 9D), suggesting that estrogen-mediated repression of EGF-induced Erk-1/-2 activity occurs at, or upstream of, Raf-1.

To further investigate the inhibitory effect of estrogen on the EGFR-to-Erk signaling pathway, we employed a Ras affinity assay to measure the ability of endogenous Ras-1 to couple to a GST fusion protein containing the Ras-binding domain of Raf-1. Raf-1/Ras complexes were detected as early as 3 min following exposure to estrogen or EGF (Fig. 9E). However, these complexes were transient and were no longer detected after 30 min of exposure to either stimulant. Cells pretreated with a diphtheria toxin mutant, CRM-197, that sequesters HB-EGF from the cell surface (40), abrogated estrogen-mediated activation of Ras, demonstrating that extracellular release of HB-EGF is necessary for estrogen-induced Ras activity. In contrast, cells exposed to estrogen for 30 min, a time interval sufficient to stimulate cAMP (Fig. 1) and restore Erk to baseline (Fig. 9E), did not block EGF-induced Ras activation yet did blunt EGF-induced stimulation of Erk (Fig. 9E).

Thus, together these data suggest that restoration of Erk-1/-2 activity to basal levels in breast carcinoma cells stimulated by estrogen or growth factor is achieved through GPR30-mediated stimulation of adenylyl cyclase, which suppresses the EGFR-to-Erk pathway through PKA-dependent inhibition of Raf-1 activity. Furthermore, these data imply that breast tumors that fail to express GPR30, or produce mutant variants of this GPCR that are unable to couple to adenylyl cyclase, may no longer be able to effectively regulate the EGFR-to-Erk pathway in response to estrogens or antiestrogens.

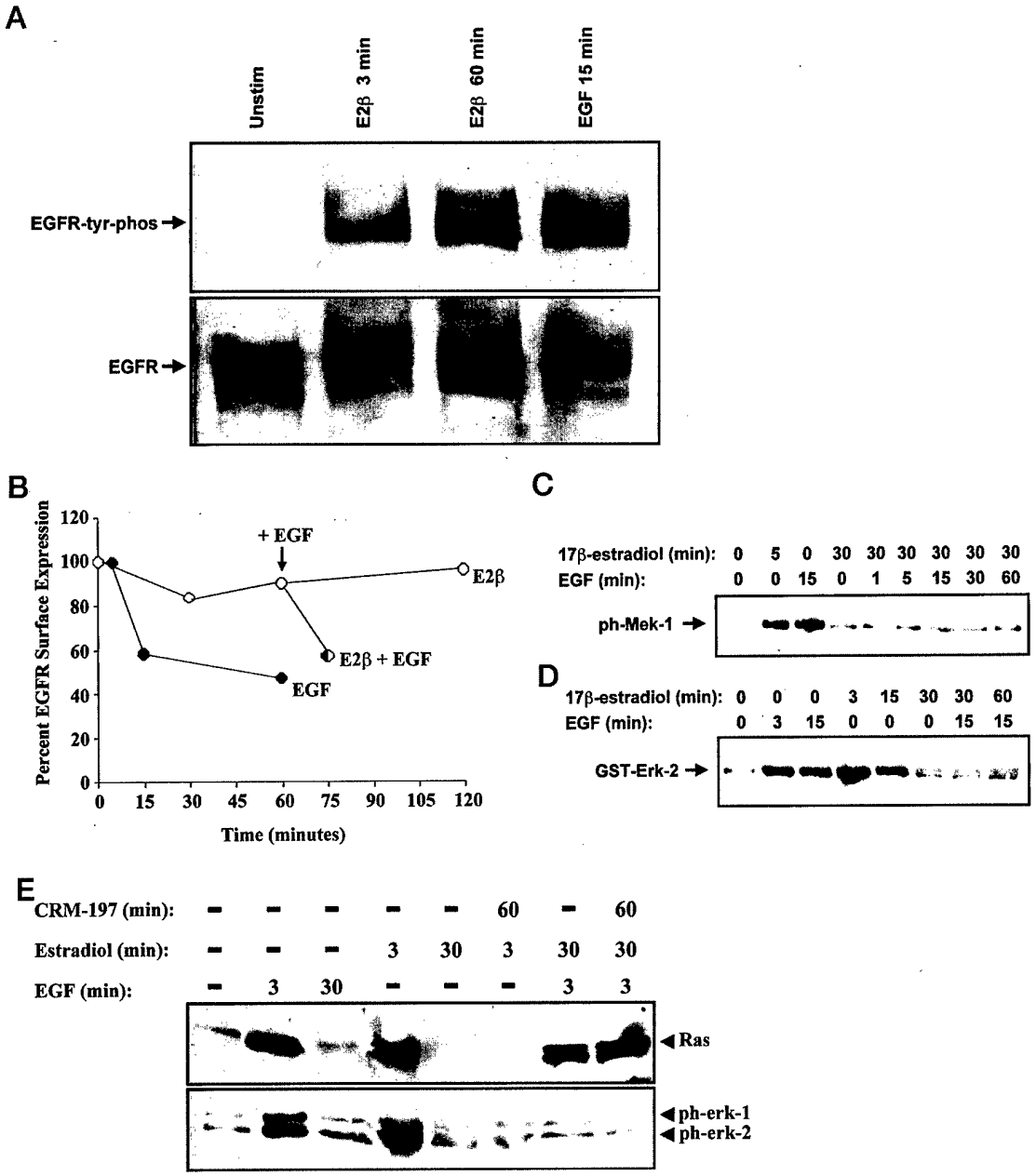

Fig. 9. Inhibition of EGF-Induced Erk-1/-2 Activity by Estrogen Occurs at the Level of Raf-1

A, Serum-deprived MDA-MB-231(GPR30) cells that were untreated or stimulated with EGF or 17β-E2 for the indicated lengths of time (minutes) were lysed in detergent. After immunoprecipitation with the ErbB1-specific monoclonal antibody, Ab-1, tyrosine-phosphorylated EGFR was detected by immunoblotting with the phosphotyrosine-specific antibody, PY20. EGFR recovery was assessed by stripping this nitrocellulose membrane and reprobing with sheep anti-EGFR antibodies. B, EGFR surface expression was assessed by flow cytometry using ErbB1-specific antibodies in MDA-MB-231 (GPR30) cells that were untreated, exposed to EGF, or pretreated with 17β-E2 before EGF stimulation. Cells were then fixed in paraformaldehyde and immunostained with the ErbB1-specific monoclonal antibody, 29.1, which reacts with an epitope external to the EGF-ligand binding domain on the receptor. Activity of Mek-1 (C) or Raf-1 (D) was measured in detergent lysates prepared from MDA-MB-231 (GPR30) cells that were untreated, EGF stimulated, or pretreated with 17β-E2 before EGF stimulation. Mek-1 activity was determined from 50 μg of total cellular protein by probing immunoblots with phospho-Mek-specific antibodies. Raf-1 activity was assessed in a cascade assay using immunopurified Raf-1, GST-Mek-1, and GST-Erk-1. Erk-1 phosphorylation was measured using phospho-specific Erk-1/-2 antibodies. (E) MDA-MB-231 (GPR30) cells were treated with EGF, 17β-E2, the diphtheria toxin mutant, CRM-197 (200 ng/ml), or combinations thereof, for the indicated times and then lysed in detergent. One milligram of cellular lysate was incubated with GST-Raf1RBD fusion protein and analyzed by Western blot for GTP-loaded Ras. P-erk expression in these samples was assessed in parallel by blotting with phospho-Erk-specific antibodies.

DISCUSSION

Estrogen exerts its effects on a diverse array of target tissues. At present, it is uncertain whether all of these effects are mediated by the known estrogen receptors, ERα and ERβ. It has long been appreciated that these ERs belong to the steroid hormone receptor superfamily and function as ligand-activated transcription factors (41). Over the past decade, a number of investigators have reported that estrogen (12, 15, 24, 42–45), and other steroid hormones (46–49) trigger rapid intracellular signaling events typically associated with membrane receptors that possess intrinsic tyrosine kinase activity or couple to heterotrimeric G proteins. Previously, we have demonstrated that estrogen acts via the GPCR, GPR30, to promote rapid transactivation of the EGFR to MAPK pathway through the release of pro-HB-EGF (23). Here, we show that through GPR30, estrogen stimulates adenylyl cyclase and inhibits Erk-1/-2 activity via a cAMP-dependent mechanism. Together these data demonstrate that estrogen signals via GPR30 to trigger opposing G protein-dependent signaling mechanisms that act to balance Erk-1/-2 activity. This mechanism of GPCR-Erk-1/-2 regulation is consistent with prior data showing a dual regulatory effect on MAPK by a single β-adrenergic receptor (50).

Here we provide several lines of evidence suggesting that estrogen-mediated activation of adenylyl cyclase occurs independently of known ERs but rather requires GPR30 protein. First, the antiestrogens, tamoxifen and ICI 182, 780, do not antagonize estrogen-induced activation of adenylyl cyclase but rather act as agonists capable of stimulating adenylyl cyclase activity (Fig. 1). Second, we show that either antiestrogens or 17β-E2 are able to promote activation of adenylyl cyclase activity in MCF-7 and SKBR3 human breast cancer cell lines that express both (37) or neither (23, 36) ERα and ERβ, respectively, but do express elevated levels of GPR30 protein. Conversely, we find that MDA-MB-231 cells that express ERβ, but not ERα and express only low levels of GPR30 protein are unable to stimulate adenylyl cyclase activity (Fig. 1B) or mediate cAMP-dependent suppression of the EGFR to MAPK pathway (Fig. 6). However, we do show that MDA-MB-231 cells forced to overexpress GPR30 are able to regulate these activities (Figs. 1C and 7) in response to estrogen.

A requirement for GPR30 in stimulation of adenylyl cyclase by estrogen is consistent with studies that have implicated GPCRs and G proteins in rapid membrane signaling events mediated by estrogen (32, 33, 45) and other steroid hormones (47–49). Our finding that antiestrogens also promote adenylyl cyclase stimulation has previously been reported by others who demonstrated that ER antagonists, namely tamoxifen and ICI 164, 384, could stimulate this activity and generate intracellular cAMP in human MCF-7 breast cells (24). These investigators also found increased levels of cAMP in the uterus of rats injected with either estrogen or the aforementioned antiestrogens. In this regard, it is noteworthy that prolonged tamoxifen use in women has been associated with endometrial hyperplasia (51) and that intrauterine injection of cholera toxin has been induces estrogen-like growth in the uterus of rats (52). Others have provided evidence that estrogen induced stimulation of adenylyl cyclase may occur via a GPCR-dependent mechanism (26, 32). These investigators have shown that SHBG, a serum protein that binds circulating estrogen and androgens with high affinity, when unliganded, specifically interacts with a membrane receptor on breast and prostate cancer cells, termed SHBGR. Upon exposure to estrogen or androgens, these preformed SHBG/SHBGR complexes bind hormone and stimulate adenylyl cyclase activity (32). Although the molecular nature of the SHBG receptor remains unknown, recent data demonstrating that: 1) nonhydrolyzable GTP analogs inhibit SHBG binding and 2) a dominant negative Gαs-subunit protein decreases estrogen-induced, SHBG-dependent cAMP signaling, indicates that this receptor may belong to the GPCR superfamily (53). Although it is possible that GPR30 may serve as a receptor for SHBG, in our experiments, as well as those conducted by others (24), no exogenous factors are required to initiate estrogen-induced activation of adenylyl cyclase. Furthermore, in contrast to the findings reported for SHBG-mediated estrogen action (25), we find that GPR30-dependent activation of adenylyl cyclase can also be promoted by the antiestrogens, tamoxifen, and ICI 182, 780 (Fig. 1C).

In other cell types, cAMP agonists are known to promote stimulation of MAPK activity via activation of the monomeric GTPase, Rap-1, which in turn, promotes B-Raf-mediated activation of Mek-1 and Erk-1/-2 (29). A similar Rap-1 dependent mechanism is activated in LNCaP prostatic carcinoma cells in response to agents that elevate cAMP (54). We have found that neither dibutyrl cAMP or cholera toxin are capable of inducing rapid activation of Erk-1/-2 in MDA-MB-231 (GPR30) cells (Fig. 2B), an effect that we show is likely due to the fact that these cells down-modulate the 95-kDa B-Raf isoform upon serum starvation (Fig. 2A). We show that estrogen-mediated repression of EGF-induced activation of the Raf-to-Erk cascade can be reversed by the cell permeant cAMP congener, KT5720 (Fig. 3). Because this analog irreversibly binds to the regulatory subunits of PKA, and thereby prevents its catalytic activation, our data indicate that estrogen mediated suppression of the EGFR-to-MAPK cascade via GPR30 occurs via PKA-dependent signaling. Other hormones and agonists that elevate cAMP are known to oppose activation of the EGFR-to-MAPK cascade in many other cell types. Several distinct PKA-dependent inhibitory mechanisms have been shown to operate. Direct phosphorylation of Raf-1 by PKA at serine residues 43 (28, 55) and 621 (56, 57) have been proposed to be responsible

for this inhibitory effect. Still others have provided evidence that PKA may act upstream of Raf-1 (58). Here we show that estrogen promotes Raf-1 inactivation (Fig. 9D), which, in turn, is associated with decreased activity of Erk-1/-2 and its activating kinase Mek-1 (Fig. 9C). Our data indicate that this estrogen action does not interfere with the ability of Ras to couple to Raf-1 *in vitro* (Fig. 9E). However, we did not explore the possibility that estrogen promotes cAMP-dependent signals via GPR30 that prevent *in vivo* coupling of Ras to Raf-1. This mechanism of GPCR- dependent inhibition of Erk has been associated with Rap-1-dependent sequestration of Raf-1 in HEK293 cells (59).

Estrogen-responsive cells employ both serum growth factors and estrogen for their growth and survival. Coordinated signaling between growth factor receptors and estrogen receptors is required for controlled growth and behavior of normal mammary epithelium. The discovery that these distinct extracellular stimuli utilize common intracellular signaling pathways, as exemplified by the EGFR-to-MAPK signaling axis, further emphasizes this concept. Several lines of evidence support the concept that the EGFR-MAPK signaling axis is a common pathway that is regulated by estrogen. EGF-related ligands enhance ER transcriptional activity (60), and this has been shown to result from MAPK-mediated phosphorylation of serine 118 within the activation function II (ATF-II) domain of the ER (10, 11). In this regard, these studies indicate that the ER lies downstream of the EGFR-MAPK signaling axis and may enhance ER-dependent cellular growth. Conversely, estrogen has been shown to increase EGFR expression and activity in the uterus (61, 62). However, it is important to note that this response is transient, and ultimately, results in the restoration of EGFR expression to levels observed before estrogen stimulation (63). Studies designed to investigate the refractoriness of ER-transfected cells to undergo estrogen-dependent proliferation have demonstrated that EGFR signaling must be silenced for estrogen-dependent proliferation to occur in these cells (64). Others have shown that estrogen can inhibit serum-mediated, MAPK-dependent growth of vascular smooth muscle cells (65).

Although our studies indicate that GPR30 may affect estrogen-mediated regulation of the EGFR-MAPK axis, others have also indicated that the ER may promote activation of MAPK (12–15). A novel functional role for the ER in rapid estrogen has also been suggested from studies that have indicated that the ER can engage and promote activation of phosphatidylinositol 3′OH kinase (66) and PKB/AKT (67). It is noteworthy that these downstream signaling effectors lie downstream of receptor tyrosine kinases, including the EGFR. Although the data presented here and previously (23) strongly suggest that GPR30 participates in the regulation of the EGFR-to-MAPK signaling axis, whether or not GPR30 acts alone or functions as part of a receptor complex remains to be determined. However, it is worth reiterating that we have demonstrated that estrogen is capable of regulating the EGFR-to-MAPK signaling axis in SKBR3 breast cancer cells that lack ERα as well as ERβ, but express GPR30 (data presented here and in Ref. 23). It is possible, however, in other cell types, GPR30 may form a signaling complex with the ER, or communicate with the ER to promote rapid nongenomic estrogen signaling.

A schematic diagram depicting a likely mechanism by which GPR30 may regulate growth factor receptor and ER signal transduction pathways is shown in Fig. 10. We have previously shown that estrogenic hormones and GPR30 act to stimulate G$\beta\gamma$-subunit protein dependent transactivation of the EGFR-to-Erk signaling axis through the release of proHB-EGF (23). Here, we demonstrate that estrogen also stimulates adenylyl cyclase activity and cAMP-dependent PKA-mediated suppression of the EGFR-Erk pathway. Our model outlines a regulatory loop comprised of opposing signals, triggered by estrogen and requiring GPR30, that serve to balance the EGFR-to-Erk pathway. Although our experiments indicate that these opposing mechanisms can be activated by estrogen *in vitro*, our results raise an interesting question regarding which one of these opposing estrogen-induced signals prevails in breast tumors *in vivo*. Amplification of EGFRs is the most common genetic alteration associated with breast cancer and is detected in 30% of all breast tumors and primarily among those tumors that fail to express ER (8). Likewise, dysregulated expression of MAPK has been reported to be a frequent event in breast cancer (16). However, mutations in Ras genes are rarely observed (less than 5% of all breast cancer cases) even though they occur frequently in other carcinomas (68). These data suggest that intermediate components of the EGFR-to-Erk cascade are tightly regulated in normal breast epithelial cells. In this regard, genetic alterations that affect signaling pathways that attenuate the EGFR-MAPK signaling cascade, including loss or mutation of GPR30, may be a common occurrence in breast cancer.

The existence of an alternative membrane-localized G protein-coupled receptor for estrogen would provide a new paradigm by which steroid hormone-activated signals interdigitate with growth factor-mediated signals to regulate the cellular behavior of steroid hormone responsive cells. Finally, the identification of GPR30 as an important mediator of estrogen action may provide further insight into the molecular mechanisms by which breast carcinomas grow and survive.

MATERIALS AND METHODS

Cell Culture

Human MCF-7 (ERα+, ERβ+), MDA-MB-231 (ERα−, ERβ+), and SKBR3 (ERα−, ERβ−) breast carcinoma cell lines were obtained from the American Tissue Culture Collection (Manassas, VA). MDA-MB-231 (GPR30) cells are stable transfectants expressing GPR30 protein and have been described previously (23). Both MCF-7 and SKBR3 cells

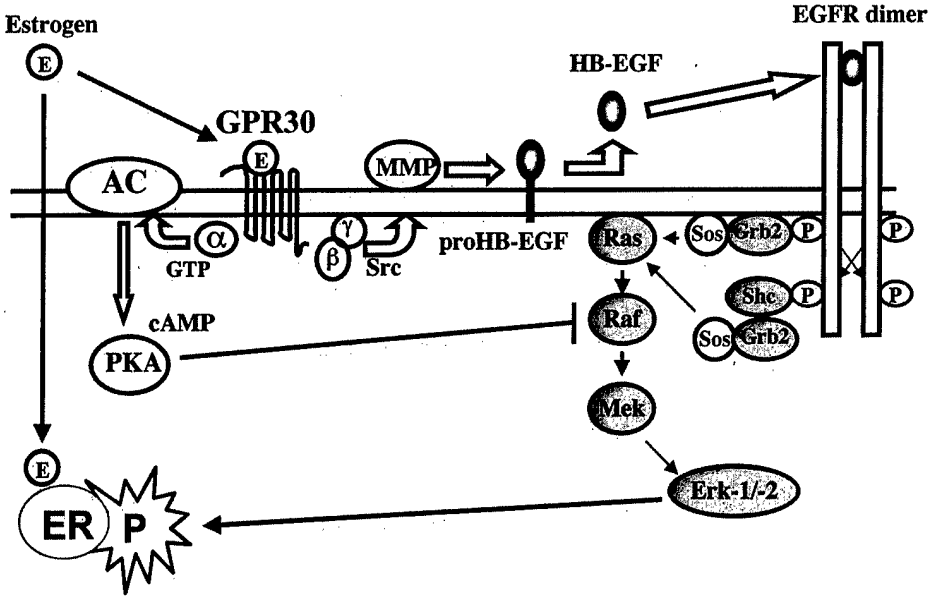

Fig. 10. Proposed Mechanism by Which Estrogen Acts via GPR30 to Regulate Growth Factor Receptor and ER Signal Transduction Pathways

Data presented here suggest that via GPR30, estrogens as well as antiestrogens are capable of stimulating adenylyl cyclase activity, which in turn, leads to PKA-mediated suppression of EGF-induced Erk-1/-2 activity. Previously, we have shown that estrogen and antiestrogens act via GPR30 to promote EGFR transactivation through a $G\beta\gamma$-subunit protein pathway that promotes Src-mediated, metalloproteinase (MMP)-dependent cleavage and release of HB-EGF from the cell surface. Thus, via GPR30, estrogen may balance Erk-1/-2 activity by stimulating two distinct G protein signaling pathways that have opposing effects on the EGFR-to-MAPK axis.

express elevated levels of GPR30 protein relative to MDA-MB-231 cells (23). All cultures were grown in phenol red-free DMEM/Ham's F12 media (1:1) supplemented with 10% fetal bovine serum and 100 μg/ml gentamicin. MDA-MB-231 (GPR30) cells were maintained in the same medium supplemented with 500 μg/ml geneticin (Sigma, St. Louis, MO).

Growth Factors, Estrogens, and Antiestrogens, cAMP Agonists, and Congeners

Recombinant human EGF was purchased from the Upstate Biotechnology, Inc. (Lake Placid, NY). Water-soluble 17β-E2; its inactive isomer, 17α-E2; progesterone; 4-hydroxytamoxifen; and cholera toxin were purchased from Sigma. The pure ER antagonist, ICI 182, 780 was obtained from Tocris Chemicals (Ballwin, MN). Dibutyrl-cAMP was obtained from Roche Molecular Biochemicals (Indianapolis, IN) and the cell permeant cAMP congener, KT5720 from Calbiochem (La Jolla, CA). The diphtheria toxin mutant, CRM-197, was purchased from Berna Products (Coral Gables, FL).

Antibodies

The p42/44 MAPK antibody that recognizes total Erk-1 and Erk-2 protein (phosphorylation state-independent) and phospho-specific antibodies that recognize either phosphorylated Erk-1 and -2 (phospho-Erk), or phosphorylated Mek-1 (phospho-Mek) were purchased from New England Biolabs, Inc., now Cell Signaling Technologies, Inc. (Beverly, MA). The Erk-2 antibodies were also purchased from the same vendor and are also known to cross react with Erk-1. Monoclonal antibodies Ab-1 (Calbiochem) and 29.1 (Sigma) recognize the ErbB1/EGFR receptor and do not cross-react with ErbB2 (Her-2/Neu), ErbB3, or ErbB4. Monoclonal antibody 29.1 recognizes an epitope external to the ligand binding domain of the EGFR and does not interfere with EGF binding. The phosphotyrosine-specific monoclonal antibody, PY20, was purchased from Transduction Laboratories, Inc., Lexington, KY). Raf-1 (C-12) antibodies raised against a peptide from the carboxyl terminus of the human Raf-1 protein were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Antibodies that recognize the 95- and 68-kDa isoforms of B-Raf (C-19) were purchased from the same vendor. Ras monoclonal antibody (clone RAS10) recognizes both the Ha- and Ki-Ras isoforms at 21 kDa and was obtained from Upstate Biotechnology, Inc.

Conditions for Cell Stimulation

Breast carcinoma cells were seeded onto 90-mm Falcon tissue culture dishes in phenol-red free DMEM/F12 medium containing 10% FCS. The following day, the cell monolayers were washed three times with phenol-red free, serum-free DMEM/F12, and exchanged for fresh phenol-red free, serum-free media on each of the following 3 d. Stimulations of quiescent cells were carried out at 37 in serum-free medium as described in the figure legends. After stimulation, monolayers were washed twice with ice-old PBS, and lysed in ice-cold RIPA buffer (150 mM NaCl, 100 mM Tris, pH 7.5, 1% deoxycholate, 0.1% sodium dodecyl sulfate, 1% Triton X-100, 3.5 mM $NaVO_4$, 2 mM phenylmethylsulfonylfluoride, 50 mM NaF, 100 mM sodium pyrophosphate plus a protease inhibitor cocktail; Complete, Roche Molecular Biochemicals). Crude lysates were clarified by centrifugation and cellular protein concentration was determined using the bichichoninic acid method according to manufacturer's suggestions (Pierce Chemical Co., Rockford, IL). Detergent lysates were stores at −70 C until use.

Western Blotting

Total cellular protein (50 μg) was boiled in standard Laemmli buffer with reducing agents and resolved by SDS-PAGE. Proteins were electrotransferred onto nitrocellulose membranes (0.45 μM pore size; Schleicher & Schuell, Inc., Keene, NH) using a semi-dry transfer cell (CBS Scientific Co., Del Mar, CA) at 1 mA/cm^2 for 4 h. Phospho-Erk was detected by probing membranes, which were preblocked in Tris-buffered saline containing 0.1% Tween-20 and 2% BSA (TBST-BSA), with phospho-Erk-specific antibodies diluted 1:1,000 in TBST-BSA for 1 h at room temperature. Rabbit antibody-antigen complexes were detected with horseradish peroxidase-coupled goat antibodies to rabbit IgG diluted 1:5,000 in TBST-BSA and visualized by enhanced chemiluminescence (Amersham Pharmacia Biotech, Arlington Heights, IL). Relative levels of total Erk-2 protein in each sample were determined by stripping the phospho-specific Erk rabbit antibodies from the nitrocellulose membrane and reprobing with antibodies to Erk-2. Phosphorylated Mek-1 protein was detected in much the same manner, except that filters to be probed with phospho-Mek antibodies were blocked in TBST containing 5% nonfat dry milk and antibodies were delivered overnight in TBST-BSA. Apparent molecular weights were determined from Rainbow molecular weight standards (Amersham Pharmacia Biotech).

Adenylyl Cyclase Activity

Cells (50 × 10^6) were homogenized in 20 ml of 10 mM Tris-HCl (pH 7.4), 5 mM EDTA samples were sonicated, and sedimented twice (1,000 × *g* for 5 min and 40,000 × *g* for 20 min). The membrane pellet was resuspended at a final concentration of 3–5 mg/ml in 75 mM Tris-HCl, pH 7.4, 2 mM EDTA, 5 mM $MgCl_2$ and stored at − 80 C. Ten micrograms of membrane protein were added to reactions containing 1 mM ATP, 50 nM GTP, 0.2 IU pyruvate kinase, 0.1 IU myokinase, 2.5 mM phosphoenolpyruvate, and 1.0 mM isobutylmethylxanthine, and treated with 17β-E2, 17α-E2, progesterone, 4-hydroxytamoxifen, or cholera toxin for 20 min at 37 C. Reactions were terminated by precipitating the samples with ice-cold ethanol. Supernatants were dried and cAMP was measured in a competitive ELISA using rabbit cAMP-specific antisera (Cayman Biochemicals, Ann Arbor, MI).

Detection of Erk-1/-2 and Raf-1 Activity

Erk-1/-2 activity was measured by standard immune complex assay utilizing myelin basic protein (MBP) as a substrate. Erk-1 and -2 were immunopurified from 500 μg of lysate using 2 μg/sample of p42/44 MAPK antibody plus 50 μl of a 50% slurry of protein G-agarose (Pierce Chemical Co.). Erk immunoprecipitates were washed twice in 50 mM HEPES (pH 7.9), 100 mM NaCl and then resuspended in immune complex kinase buffer: 25 mM HEPES, pH 7.9, 1 mM DTT, 10 mM cold ATP, 50 μM ^{32}P-γ-ATP (0.25 μCi), and 8 μg MBP (Upstate Biotechnology, Inc.). After a 30-min incubation at 30 C, samples were boiled in standard Laemmli buffer and subjected to SDS-PAGE. Gels were dried and exposed to Kodak XAR film for autoradiography. Raf-1 activity using a kinase cascade assay kit, essentially as described by the manufacturer (Upstate Biotechnology, Inc.). Raf-1 was immunoprecipitated from 500 μg of lysate using 2 μg/sample of Raf-1 antibody plus 50 μl of a 50% slurry of protein G-agarose. Raf-1 immunoprecipitates were washed three times in assay dilution buffer (20 mM MOPS, pH 7.2; 25 mM β-glycerol phosphate, 5 mM EGTA, 1 mM sodium orthovanadate, and 1 mM dithiothreitol) and then resuspended in the same buffer containing 1 mM ATP, 75 mM $MgCl_2$ and 0.4 μg of unactivated (unphosphorylated) Gst-Mek1 protein. After a 30-min incubation at 30 C, 1.0 μg of unactivated (unphosphorylated) Gst-Erk2 was added to this kinase reaction and incubated an additional 30 min at the same temperature. The reaction was terminated by the addition of boiling standard Laemmli buffer. Products of the reaction were separated by SDS-PAGE and phosphorylated GST-Erk2 was detected by immunoblotting sing phospho-Erk-specific antibodies as described above.

Affinity Assay for Ras Activation

Serum-starved cells were stimulated at 37 C for indicated times and then immediately lysed in ice-cold MLB lysis buffer (25 mM HEPES, pH 7.5, 150 mM NaCl, 1% Igepal CA-630, 0.25% sodium deoxycholate, 10% glycerol, 25 mM NaF, 10 mM $MgCl_2$, 1 mM EDTA, 1 mM sodium orthovanadate, 10 μg/ml leupeptin, 10 μg/ml aprotinin). Per the manufacturer's specifications, activated Ras was isolated from these lysates using GST-Raf1BD coupled to glutathione agarose beads (Upstate Biotechnology, Inc.). Proteins were eluted from the beads by boiling in 2× Laemmli buffer, resolved through 12% SDS-polyacrylamide gels and transferred to nitrocellulose. Membranes were then blocked in PBS containing 0.05% Tween 20 and 5% nonfat dried milk and probed with a Ras monoclonal antibody (clone RAS10) overnight at 4 C. Ras antibodies were detected using horseradish peroxidase-coupled antimouse secondary antibodies and a chemiluminescent substrate.

Detection of Phosphotyrosyl Residues on the EGFR

Tyrosine phosphorylation of the EGFR was assessed by immunoblotting EGFR immunoprecipitates with phosphotyrosine-specific antibodies. EGFR was immunoprecipitated from 250 μg of total cell protein, extracted in RIPA buffer using 2 μg/sample of the ErbB1-specific monoclonal antibody, Ab-1. EGFR-Ab-1 complexes were precipitated with 50 μl of a 50% slurry of protein G-agarose (Pierce Chemical Co.). EGFR immunoprecipitates were washed, resuspended in standard Laemmli buffer containing reducing agents, and subjected to SDS-PAGE. After electrophoresis, the immunoprecipitated material was then transferred to nitrocellulose membranes, blocked with TBST-BSA, and then immunoblotted with the phosphotyrosine-specific monoclonal antibody, PY20.

EGFR Internalization

Serum-deprived MDA-MB-231 (GPR30) cells were detached in HEPES-buffered saline containing 5 mM EDTA, washed twice in phenol red-free DMEM/F12 containing 0.5% BSA and resuspended at a concentration of 10^6/ml in the same buffer in the absence of BSA. One million cells were aliquoted into flow cytometry tubes and allowed to equilibrate to 37 C in a water bath for 15 min. Samples were either untreated or exposed to 1 nM 17β-E2 or 10 ng/ml of EGF for various lengths of time at 37 C. After stimulation, cells were fixed by adding an equal volume of 8% paraformaldehyde to each sample. Cells were collected by centrifugation, washed twice in PBS-containing 0.5% BSA (PBS-BSA) and resuspended in the same. Fixed cells were incubated with 5 μg/ml EGFR mAB 29.1 for 30 min at room temperature. Cells were then washed twice in PBS-BSA, resuspended in the same buffer containing a 1:250 dilution of fluorescein isothiocyanate-conjugated antimouse IgG antibodies, and incubated for 30 min at room temperature. Cells were then centrifuged, washed, and surface expression was assessed by flow cytometry using a FACScan instrument.

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Epidermal growth factor receptor (EGFR) transactivation by estrogen via the G-protein-coupled receptor, GPR30: a novel signaling pathway with potential significance for breast cancer

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Abstract

The biological and biochemical effects of estrogen have been ascribed to its known receptors, which function as ligand-inducible transcription factors. However, estrogen also triggers rapid activation of classical second messengers (cAMP, calcium, and inositol triphosphate) and stimulation of intracellular signaling cascades mitogen-activated protein kinase (MAP K), PI3K and eNOS. These latter events are commonly activated by membrane receptors that either possess intrinsic tyrosine kinase activity or couple to heterotrimeric G-proteins. We have shown that estrogen transactivates the epidermal growth factor receptor (EGFR) to MAP K signaling axis via the G-protein-coupled receptor (GPCR), GPR30, through the release of surface-bound proHB-EGF from estrogen receptor (ER)-negative human breast cancer cells [Molecular Endocrinology 14 (2000) 1649]. This finding is consistent with a growing body of evidence suggesting that transactivation of EGFRs by GPCRs is a recurrent theme in cell signaling. GPCR-mediated transactivation of EGFRs by estrogen provides a previously unappreciated mechanism of cross-talk between estrogen and serum growth factors, and explains prior data reporting the EGF-like effects of estrogen. This novel mechanism by which estrogen activates growth factor-dependent signaling and its implications for breast cancer biology are discussed further in this review.

Keywords: Breast cancer; G-protein-coupled receptor; Epidermal growth factor receptor

1. Introduction

Estrogens induce diverse physiological effects. Their actions are required for normal development and growth of female reproductive tissues and in certain cases, promote the growth of tumors that arise from these tissues. In addition to their impact on female reproductive tissue, estrogens regulate bone integrity [1], cardiovascular function [2] and the central nervous system [3]. These physiological and pathophysiological responses are manifested by specific receptors whose identity has important implications for human health and disease. However, the fact that estrogens promote a multitude of biochemical actions, some of which occur within seconds, others of which are measured over several hours, indicates that more than one class of receptor may participate in estrogen signaling.

The first known receptor for estrogen, termed estrogen receptor (ER), was described based on its specific binding activity in extracts prepared from rat uterus and vagina [4]. Since then its protein sequence has been determined [5] and its three-dimensional molecular structure resolved [6]. Based on its homology to receptors for other steroid hormones, the ER is classified as a member of the steroid hormone receptor (SHR) superfamily, which collectively functions as hormone-inducible transcription factors [7]. Transcriptional activity of the ER is regulated by allosteric alterations in its structure induced by estrogen and cofactors that associate with the ER. The molecular details concerning *cis* and *trans* regulation of ER functionality have been reviewed elsewhere [8,9]. Further complexity regarding ER signaling has been provided by the discovery of ER-related proteins. The first of these to be described, ERβ, was isolated from human prostate tissue and has also been shown to facilitate estrogen-mediated gene transcription [10]. Last year, a third, more distantly related member of the ER family, ERγ, was cloned in teleosts [11]. This newest member of the ER family exhibits an expression pattern distinct from that observed for ERα and ERβ [11]. These findings provide evidence that at least three SHRs may act in concert to promote the effects of estrogen.

It has long been suspected that other receptors, distinct from the ER, may participate in estrogen signaling. This theory is borne from the observation that in addition to its

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ability to promote gene transcription, estrogen stimulates classical second messengers, including cAMP [12,13], inositol phosphate [14], and calcium [15,16]. More recently, it has been shown that estrogen also triggers signaling cascades typically linked to membrane receptors that possess tyrosine kinase activity or couple to heterotrimeric G-proteins, such as mitogen-activated protein kinase (MAP K) [17–19], phosphatidylinositiol 3-OH kinase and AKT/protein kinase B [20–22]. These latter effects of estrogen occur more rapidly (within seconds to minutes) than gene transcription events that are attributed to the ER (over the course of several hours). Moreover, unlike ER-mediated gene transcription, estrogen-induced second messenger signaling is insensitive to inhibitors of gene transcription. Due to the fact that heterotrimeric G-proteins have been shown to be required for estrogen-induced second messenger activation, others have proposed that estrogen may signal via a G-protein-coupled receptor (GPCR) [23–25]. Still others have provided evidence that ER-related proteins are associated with rapid estrogen signaling from the plasma membrane and this topic has been reviewed elsewhere [26,27].

2. Transactivation of the epidermal growth factor receptor (EGFR) by estrogen

2.1. Background and historical perspective

Both estrogen and EGF are required for the growth and survival of estrogen responsive tissues. While these extracellular stimuli are structurally distinct, they exert physiological effects that overlap. For instance, both estrogen and EGF act as potent mitogens for cells from mammary epithelia and uterine endometrium [28]. However, the receptors that mediate the effects of estrogen and EGF utilize seemingly divergent signaling mechanisms. The proliferative effects of estrogen are primarily mediated by the ER and have been linked to its ability to induce gene transcription in these tissues [29]. In contrast, the biological effects of EGF are transmitted through transmembrane receptor tyrosine kinases (RTKs), known as EGFRs, which signal via their ability to recruit intracellular signaling cascades.

EGFR (ErbB1/HER-1) is the prototypical member of a family of four structurally-related RTKs. The other members include: ErbB2/HER-2, ErbB3/HER-3, and ErbB4/HER-4 and together they have been shown to play an integral role in the development and growth of the mammary gland and uterus [30,31]. Individual EGFRs recognize members of a family of small polypeptide ligands that are homologous to EGF (amphiregulin; betacellulin; heparan-bound EGF, HB-EGF; neuregulins; and transforming growth factor alpha, TGF-α). The exception to this rule is ErbB2/HER-2, for which no known physiological ligand exists. It should be noted that while EGFR ligands are found in serum, they are synthesized as nascent, inactive membrane-anchored precursors that must be cleaved and released by metalloproteinases to generate the active, mature form of the growth factor [32]. Upon binding their cognate ligands, EGFRs form homodimers and heterodimers which results in the activation of their intrinsic kinase activity and autophosphorylation of specific tyrosine residues within their cytoplasmic domains [33]. These phosphotyrosine residues, in turn, serve as nucleation sites for the recruitment of signal transduction complexes. Coupling of these complexes to the activated EGFR is mediated by phosphotyrosine binding motifs, known as SH2 domains. Primary signal transducers may link directly to the activated EGFR, as is the case for phospholipase Cγ. Alternatively, effectors with enzymatic activity may be bridged to the activated EGFRs via adaptor proteins, such as Shc, Grb-2, the p85-subunit of PI3K, and Gab1. Activated EGFR has been shown to recruit molecular signaling complexes that stimulate MAP K, PI3K, AKT/protein kinase B.

Data showing that estrogen activates intracellular signaling events similar to those activated by EGF suggests these ostensibly divergent signaling mechanisms may cross-communicate. For example estrogen activates the mitogen-activated protein kinases, Erk-1 and Erk-2, signaling intermediaries that lie downstream of the EGFR. MAP K as a signaling node utilized by estrogen and EGF is supported by prior data that showed that the ability of EGF to augment estrogen-induced cellular proliferation is linked to MAP K-mediated phosphorylation of the ER [34,35]. More recent work has shown that estrogen also activates a variety of signaling networks that are coupled to EGFRs, including phosphatidylinositol 3-OH, AKT/protein kinase B, and endothelial nitric oxide synthase [20,21,36,37]. These findings support earlier work that indicated interplay between estrogen and EGF. In vivo administration of estrogen had been shown to upregulate EGFR expression [38,39]. While this was shown to be the consequence of ER-mediated gene transcription, other reports indicated that estrogen could promote rapid EGF-like effects. For example intrauterine injection of estrogen increased the local concentration of EGF [40] and induced tyrosine phosphorylation of the EGFR [41]. In this latter study, it was also shown that angiotensin II induced EGFR autophosphorylation in vitro. This finding was considered a novelty at this time since it was already known that neither estrogen nor angiotensin II serves as a ligand for the EGFR. Recently, it has been demonstrated that angiotensin II transactivates the EGFR through intracellular signals that are transduced via its GPCR [42]. Cross-talk between GPCRs and EGFRs is not unique to angiotensin II. In fact, many ligands that employ GPCRs, namely endothelin, thrombin, carbachol, and lysophosphatidic acid, in part, transmit intracellular signals via their ability to transactivate EGFRs [43].

Heterotrimeric G-proteins have been implicated in second messenger signaling by estrogen [23,25,44] and thus, GPCRs serve as likely candidates to facilitate estrogen-induced second messenger signaling. GPCRs transduce their signals via G-protein heterotrimers (αβγ) that dissociate into free Gα-subunit protein and Gβγ-subunit protein complexes following ligand stimulation [45]. Classical second

messenger signaling is initiated by membrane-associated enzymes and ion channels that are regulated by Gα-proteins, and for these reasons, they have been implicated in rapid estrogen signaling [23–25]. In contrast, GPCR-mediated EGFR transactivation often occurs via Gβγ-subunit signaling [46]. In that a single GPCR agonist can simultaneously promote both Gα- and Gβγ-dependent signaling [47], the hypothesis that a GPCR may participate in rapid estrogen signaling is particularly attractive since it provides a singular mechanism by which both second messenger signaling and EGF-like effects occur.

2.2. A role for the G-protein coupled receptor homologue, GPR30, in EGFR cross-talk

GPR30, has been cloned by several laboratories [48–53] and has been referred to as FEG-1, CMKRL2, CEPR, and LyGPR. Its deduced amino acid sequence indicates that it exhibits a serpentine, heptahelical structure that is characteristic of the GPCR superfamily. By structural homology, GPR30 most closely resembles receptors for angiotensin II, chemokines and other peptide ligands. Due to this homology, prior studies suggested that the ligand for this receptor homologue is possibly a peptide. An assortment of chemotactic peptides, including IL-8, GRO-α, MCP-1, MCP-3, MIP-1a, C3a, C5a, RANTES, LTB-4 and other peptide ligands, such as angiotensin II and angiotensin IV, have been screened and shown not to bind to GPR30 [48,50]. GPR30 is widely expressed and its mRNA is found in breast, heart, leukocytes, brain and vascular endothelium [48–53]. These tissues are responsive to the effects of estrogen and it has been noted that this expression pattern is consistent with the ability of GPR30 to function as a hormone or neurotransmitter.

Based on the observations that GPR30 is preferentially expressed in ER-positive relative to ER-negative breast tumor cell lines [49] and that inhibitors of G-protein signaling block second messenger signaling by estrogen [14,24], we queried whether GPR30 may participate in rapid signaling by estrogen. We found this possibility particularly intriguing because others had previously demonstrated that estrogen-induced adenylyl cyclase activity in MCF-7 breast cancer cells that express GPR30 mRNA but did not stimulate this activity in MDA-MB-231 cells that express little GPR30 mRNA [13]. A similar response pattern is observed in these two breast cancer cell lines regarding their ability to activate MAP K following exposure to estrogen. MCF-7 cells undergo estrogen-induced MAP K activation, while MDA-MB-231 cells do not [44]. Others have concluded that the ability to trigger estrogen-induced Erk activity in MCF-7 and other cell types is dependent upon ER-like proteins [17,54]. However, we find that estrogen-induced Erk activation occurs in human SKBR3 breast cancer cells that fail to express either ERα or ERβ [55] but make GPR30 protein [44]. Yet, this response is not measured in MDA-MB-231 cells that express ERβ but little GPR30 protein. Upon transfection with a GPR30 cDNA, MDA-MB-231 cells overexpress GPR30 protein and acquire the capacity to promote Erk activation in response to 17β-estradiol. GPR30-dependent Erk activation is also induced by ER antagonists, including ICI 182, 780, but not by 17α-estradiol or progesterone [44]. This result provides further evidence that this estrogen action occurs independent of the ER. Moreover, this finding is consistent with data by others demonstrating that pure antiestrogens, such as ICI 164, 384, function as agonists with regards to their ability to stimulate adenylyl cyclase activity in MCF-7 cells [13]. In contrast, others have shown that antiestrogens block estrogen-induced Erk activation [17,54]. One likely source of this discrepancy is the timing between the addition of the antiestrogen and estrogen and the measurement of Erk activity. We find that simultaneous presentation of estrogen and antiestrogen does not inhibit Erk activation. In contrast, cells previously exposed to antiestrogen become refractory to other stimuli that activate Erk-1/-2, including estrogen or EGF (Filardo and Quinn, unpublished data).

Unlike RTKs, GPCRs signal to Erk via a number of distinct signaling pathways, some of which require monomeric GTPases, such as Ras or Rap, others activate Raf or Mek directly [56]. In some instances, GPCR stimulation leads to the activation of Src-related tyrosine kinases and the assembly of Grb-2/Sos/Shc complexes on the EGFR. In conjunction with the finding that Src can directly phosphorylate the EGFR [57], these observations suggest the possibility that GPCRs may activate EGFRs via Src-mediated phosphorylation of the EGFR cytoplasmic tail. Consistent with this idea, we have shown that GPR30-dependent, estrogen-induced Erk activation occurs via Gβγ-subunit protein signaling and downstream activation of Src-related tyrosine kinases ([44]; see Fig. 1). This is evidenced by the fact that pertussis toxin, Gβγ-subunit sequestrant peptides, and Src-related tyrosine kinase inhibitors all act to blunt estrogen-induced activation of Erk. In contrast, none of these inhibitors adversely affect EGF-stimulated Erk activity in breast cells [44]. While this result suggests that Gβγ-subunit proteins and EGFRs utilize distinct mechanisms to stimulate Erk, both signal via Ras-dependent Erk activation [58]. More recent data indicates that the conversion point between Gβγ-subunit protein and EGFR signaling lies upstream of Ras at the level of the EGFR [46,59]. Recently, it has been shown that some GPCRs mediate transphosphorylation of the EGFR and downstream signaling via metalloproteinase-dependent cleavage and release of heparan-bound EGF [43]. Similarly, we have shown that estrogen signaling to Erk is dependent upon transactivation of the EGFR via the release of surface-associated HB-EGF [44]. In support of this concept, we find that estrogen signaling to Erk can be blocked by: (i) specific inhibitors of EGFR tyrosine kinase, (ii) neutralizing HB-EGF antibodies, and (iii) downmodulation of proHB-EGF from the cell surface by the diphtheria toxin mutant, CRM-197. The fact that these inhibitors completely abrogate estrogen-induced EGFR receptor tyrosine phosphorylation indicates that Src must act upstream of HB-EGF

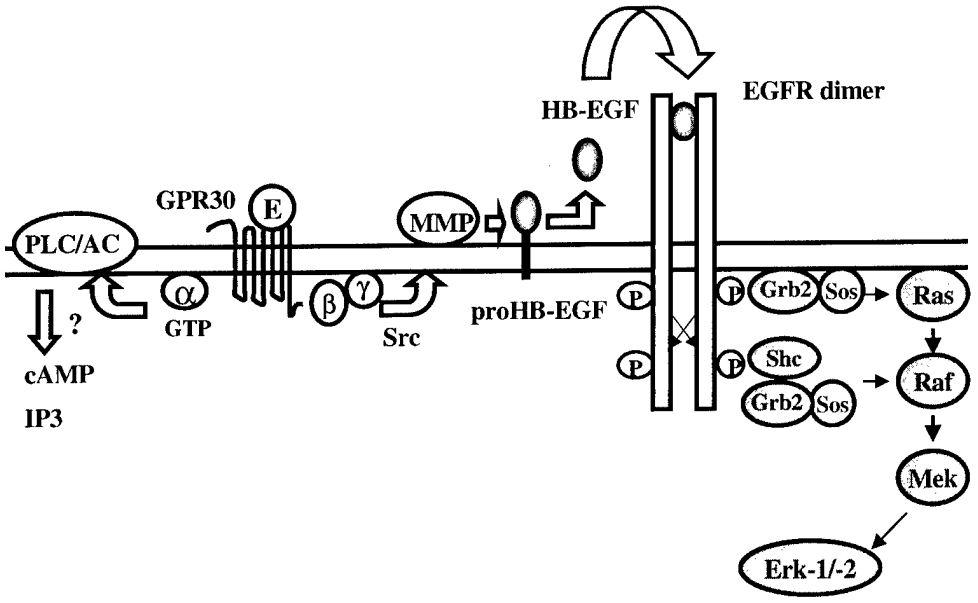

Fig. 1. Transactivation of the EGFR by estrogen via the G-protein coupled receptor, GPR30, estrogen activates Gβγ-subunit/Src family kinase-dependent intracellular signals that promote the release of nascent proHB-EGF from the cell surface. Free, active HB-EGF binds to the EGFR (ErbB1) and facilitates receptor dimerization and downstream activation of the mitogen-activated protein kinases, Erk-1 and -2.

release and can not directly phosphorylate the EGFR. Activation of an HB-EGF autocrine loop via GPR30-dependent, estrogen signaling provides a novel mechanism by which estrogen may promote EGF-like effects.

More than one GPCR may promote rapid estrogen signaling. The membrane receptor for sex hormone binding globulin (SHBG), a plasma protein that binds estrogen, provides one such possibility. Via the SHBG receptor, estrogen promotes Gαs-mediated activation of adenylyl cyclase [60]. However, the SHBG receptor appears to be distinct from GPR30. First, exogenous SHBG is not required for GPR30-dependent activation of the EGFR to Erk signaling axis [44]. Secondly, while antiestrogens promote GPR30-dependent transactivation of the EGFR, they do not function as agonists for SHBG-mediated stimulation of adenylyl cyclase. While these observations indicate that more than one GPCR may participate in rapid estrogen signaling, it is likely that further complexity in estrogen-mediated GPCR signaling may occur due to coupling of different G-protein heterotrimers with the same receptor. For example while Gαq-, Gαs-, and Gαi-coupled receptors signal to Erk-1/-2, they may also promote signals in parallel that are independent of Erk-1/-2 activation [56]. For example estrogen regulates inositol phosphate [14], as well as calcium mobilization [54] or influx [25]. Others have noted that mobilization of intracellular calcium precedes MAP K with no apparent increase in inositol triphosphate in human MCF-7 breast cancer cells [54]. We did not measure whether or not calcium was required for estrogen-induced transactivation of the EGFR or downstream activation of Erk. Evidence exists indicating that via its GPCR, angiotensin II may promote either calcium-dependent [61] or -independent [62] EGFR transactivation in different cell types. Thus, it is important to consider whether GPR30-mediated EGFR transactivation and second messenger signaling may be cell context specific. The observation that cell activation status promotes differential coupling of heterotrimers to the same receptor [63], further suggests this possibility.

Transactivated EGFRs recruit signaling complexes other than the Ras-to-Erk pathway. For example the EGFR tyrosine kinase inhibitor, AG-1478, abrogates the ability of lysophosphatidic acid to mediate Gβγ-subunit protein-dependent activation of PI3K [64]. In this regard, it is noteworthy that estrogen-induced activation of PI3K has been observed in HUVECs [21] and that these cells express GPR30 [53]. Nitric oxide production by HUVECs has been associated with a signaling pathway that involves PI3K and AKT-mediated phosphorylation of eNOS [21]. It is important to point out, however, that PI3K does not necessarily lie downstream of the EGFR and that both Gαi- and Gαq-coupled receptors have been shown to signal directly to PI3K [65,66]. Heterodimerization of EGFRs provides another means to extend the effects of GPCR-mediated transactivation because it is known that each EGFR family member has unique signaling properties. ErbB3 is a particularly interesting example, since this receptor encodes multiple binding sites for the regulatory subunit of PI3K and thus, recruits PI3K efficiently; yet, this receptor lacks tyrosine kinase activity [67]. In contrast, canonical binding sites for the regulatory subunit of PI3K are not found on ErbB1, however, this receptor stimulates PI3K [68]. Upon presentation with EGF ligands, cells that coexpress ErbB1 and ErbB3, form heterodimers which efficiently recruit PI3K [69]. More recently, it has been appreciated that

ErbB1 employs the docking protein Gab1 to recruit PI3K and thereby provides a mechanism to recruit this signaling enzyme in cells that lack ErbB3 [70,71]. Additionally, the ErbB1 adapter, Shc, recruits PI3K by assembly of a Shc–Grb2–Gab2–PI3K complex [72].

3. Significance for breast cancer biology

Estrogen induces EGF-like effects in vivo [28] and prior data has indicated that the EGFR may be a vehicle for estrogen action. Approximately, a decade ago, it was demonstrated that intrauterine administration of estradiol resulted in increased concentrations of EGF [40] and EGFR autophosphorylation [41]. Further, evidence of a relationship between the EGFR and estrogen was provided by data showing that neutralizing antibodies to EGF inhibited estrogen-mediated proliferation in the uterus [73]. Our data indicates that estrogen-mediated transactivation of the EGFR occurs independently of the ER and requires GPR30 [44]. This novel mechanism of estrogen action may have profound implications with regards to our understanding of the biology and treatment of breast cancer.

Amplification of EGFR family members is the most common genetic alteration associated with breast cancer. Overexpression of HER-2/neu occurs in approximately one-third of all breast tumors, the majority of which fail to express ER [74]. However, its importance in breast cancer may occur early in disease, as indicated by the fact that elevated HER-2/neu is observed in roughly 60% of all cases of ductal carcinoma in situ (DCIS) [75]. Since the majority of DCIS occurs in women prior to menopause, it has been speculated that the expansion of precancerous cells is the result of mitogenic coupling between EGF and estrogen. In that overamplified EGFRs undergo ligand-dependent signaling responses. This hypothesis is particularly interesting, especially in light of the fact that elevated concentrations of EGF ligands and matrix metalloproteinases [76] have been detected in breast cancer. In this regard, it is tempting to speculate that estrogen may promote EGFR transactivation events in vivo. Based on the fact that EGFRs extend their signaling capacity due to their ability to form heterodimers, and that all four EGFRs are expressed in mammary epithelia, GPR30-mediated transactivation of the EGFR may have particular significance for breast cancer. ErbB2 provides an interesting example of possible cross-signaling between EGFR members. ErbB2, is an orphan receptor that has no known physiological ligand. However, ErbB2 is strongly activated through its interactions with other EGFRs and it is favored over other heterodimers or homodimers [77]. HER-2 has been shown to increase both the amplitude and duration of MAP K activation by EGF ligands [78]. In this regard, recruitment of HER-2 by GPR30 provides a possible mechanism by which estrogen couples to the EGFR-to-MAP K signaling pathway. Frequent detection of elevated levels of phosphorylated MAP K in breast cancer is consistent with the hypothesis that hyperactivation of the EGFR-to-MAP K signaling axis is achieved by both estrogen-dependent and -independent mechanisms [79].

Full activation of ER transcriptional activity requires MAP K-mediated phosphorylation at serine 118 within the ATF-I domain of the ER. Phosphorylation at this site is promoted by EGF [34,35] and estradiol [80]. Prior observations indicate that estrogen promotes, in parallel, second messengers, activation of intracellular signaling enzymes, and gene transcription. Our data indicates that via GPR30, estrogen may, in turn, regulate or "prime" the transcriptional activity of the ER. Constitutive Erk activation achieved as a result of mutation or excessive growth factor stimulation may result in chronic phosphorylation of serine 118 of the ER, thereby facilitating hyperactive estrogen-dependent tumor cell proliferation. In this regard, it is interesting to consider that breast tumors that express low levels of ER, yet maintain GPR30, may maintain some ER function as a consequence of extrinsic or intrinsic events that hyperactivate Erk. EGFR transactivation via GPR30 provides a mechanism by which ER-negative tumors that maintain GPR30 expression may remain responsive to estrogen.

The presence of the ER is the most important parameter in predicting improved disease-free survival and responsiveness to antiestrogen therapy [81]. These clinical data support research studies that have demonstrated that antiestrogens act as ER antagonists by competitively blocking estrogen binding sites on the ER [82]. Still, one in four patients with ER-positive tumors do not respond favorably to antiestrogens, while one in six patients with ER-negative tumors undergo objective tumor regression following antiestrogen therapy [81]. These observations indicate an alternative mechanism for estrogen action. Further support for this concept comes from the fact that prolonged tamoxifen use is associated with endometrial hyperplasia [83] and that this antiestrogen, and others, also behave as agonists in vitro [13,44,84,85]. Our finding that the antiestrogens, tamoxifen and ICI 182, 780 promote GPR30-dependent transactivation of the EGFR is consistent with studies showing that steroids and their antihormones may act through membrane receptors and heterotrimeric G-proteins [25,86,87]. Future studies will define the role of GPR30 in breast cancer biology. These efforts will also determine its value in refining our ability to predict responsiveness to antiestrogen therapy and will determine whether GPR30 constitutes a valuable therapeutic target in breast cancer.

4. Conclusions

Estrogen triggers rapid activation of classical second messengers and intracellular signaling events that lie downstream of EGFRs. The recent recognition that GPCRs transduce signals, in parallel, which stimulate second messengers and activate EGFRs, suggests that GPCRs are well-suited as candidates to facilitate nongenomic estrogen signaling.

Our data demonstrates that the orphan receptor GPR30 may serve such a role. We have reported that independent of ERα and ERβ, estrogen transactivates the EGFR-to-Erk signaling axis via GPR30-dependent activation of an HB-EGF autocrine loop. Our data implies that GPR30 may have particular significance for the growth and survival of estrogen-negative breast tumors. Breast tumors that fail to express ER but maintain GPR30-dependent EGFR transactivation may remain estrogen responsive by employing growth factor dependent intracellular signaling pathways.

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